# The Evolution of Sexually Selected Traits

## Comparative Morphology and Chemistry of the Cephalic Glands of Beewolves (Hymenoptera, Crabronidae)


Dissertation zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Fakultät für Biologie und Vorklinische Medizin
der Universität Regensburg

vorgelegt von
**Katharina Weiß**

aus Amberg

im Jahr 2017

# The Evolution of Sexually Selected Traits

## Comparative Morphology and Chemistry of the Cephalic Glands of Beewolves (Hymenoptera, Crabronidae)


Dissertation zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Fakultät für Biologie und Vorklinische Medizin
der Universität Regensburg

vorgelegt von
**Katharina Weiß**

aus Amberg

im Jahr 2017

Das Promotionsgesuch wurde eingereicht am**:**

Die Arbeit wurde angeleitet von: **Prof Dr. Erhard Strohm**

Unterschrift:

“I can entertain no doubt, after the most deliberate study and dispassionate judgment of which I am capable, that the view which most naturalists entertain, and which I formerly entertained - namely, that each species has been independently created - is erroneous.”

*Charles Darwin, ‘The Origin of Species’ (1859)*

“Intelligent life on a planet comes of age when it first works out the reason for its own existence. […] Living organisms had existed on earth, without ever knowing why, for over three thousand million years before the truth finally dawned on one of them. His name was Charles Darwin.”

*Richard Dawkins, ‘The Selfish Gene’ (1976)*

## CONTENTS

## LIST OF PUBLICATIONS

This PhD thesis is based on the following manuscripts.

**Weiss K, Strohm E, Kaltenpoth M, Herzner G.** (2015) Comparative morphology of the postpharyngeal gland in the Philanthinae (Hymenoptera, Crabronidae) and the evolution of an antimicrobial brood protection mechanism. *BMC Evolutionary Biology*, doi: 10.1186/s12862-015-0565-0. **(Chapter 2)**

**Weiss K, Strohm E, Herzner G.** (*in preparation*) Evolution and phylogenetic distribution of antimicrobial brood defense in beewolves (Hymenoptera, Crabronidae): Evidence from postpharyngeal gland chemistry. **(Chapter 3)**

**Weiss K, Strohm E, Schmitt T, Wurdack M, Herzner G.** (*in preparation*) Does the morphology of the postpharyngeal gland reflect potential functional changes in bee- *vs.* beetle-hunting species of the digger wasp genus *Cerceris* (Hymenoptera, Crabronidae)? **(Chapter 4)**

**Weiss K, Herzner G, Strohm E.** (2017) Sexual selection and the evolution of male pheromone glands in philanthine wasps (Hymenoptera, Crabronidae). *BMC Evolutionary Biology*, doi: 10.1186/s12862-017-0963-6 **(Chapter 5)**

# CHAPTER 1

# GENERAL INTRODUCTION

Evolution is defined as the change of allele frequencies in a gene pool across generations. It will inevitably occur provided that there is variation among individuals in a population and that this variation is, at least partly, heritable. The result is the non-random survival and reproduction of individuals, leading to the gradual change of the population over time. This process is called natural selection. The concept of evolution by natural selection was first outlined by Charles Darwin in his seminal work '*On the Origin of Species*' (Darwin 1859). Generally, natural selection leads to adaptations that maximize the fitness of their bearers in a given environment.

Yet, as already noted by Darwin (1859, 1871), often the most prominent traits observed in animals, like gaudy coloration, elaborate songs, and ornaments, may hardly be suited to enhance fitness under natural selection. These traits are attributed to sexual selection, a form of selection that maximizes the attractiveness (i.e. reproductive success) of their bearers, often even at the expense of survival. Today, sexual selection is often assumed to promote rapid evolutionary changes and drive population divergence and speciation (e.g. Seehausen & van Alphen 1999, Kraaijeveld et al. 2011, Wagner et al. 2012, Seddon et al. 2013; but see e.g. Huxley 1938, van Doorn et al. 2004).

The present PhD thesis aims to establish the digger wasp subfamily Philanthinae (Hymenoptera, Crabronidae) as a model for the study of trait evolution under natural and sexual selection. We assess the interspecific variation in the exocrine head glands of these wasps that are under natural selection in females, but under sexual selection in males. We compare interspecific variation in gland morphology and chemistry within and between males and females and interrelate our results to ecological aspects. Using the glands of females as a 'model under natural selection', we are able to gain more direct access to the contribution of sexual selection in the evolution of interspecific diversity in male head glands.

## 1.1 Sexual selection and the evolution of interspecific variation

### 1.1.1 Darwin's unresolved problem: The asymmetry of sexual selection

*"The sight of a feather in a peacock's tail, whenever I gaze at it, makes me sick!"*
*Charles Darwin, Letter to Asa Gray (3 April 1860)*

One of the main obstacles to Darwin's theory of evolution by natural selection was the existence of elaborate traits that occur only in one sex and do not seem to be conducive to the fitness of their bearers under natural selection, like the enormous tail of a peacock. To explain the evolution of such traits, Darwin proposed sexual selection as a distinct selective force that "depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction" (Darwin 1871, pp. 254-255). Yet, explaining the evolution of sexually selected traits presented Darwin with some difficulty since he could not satisfactorily explain (1) why non-random mate choice has evolved in the first place and (2) why it is mostly asymmetric, with males showing pronounced secondary sexual characters, like colorful plumage, elaborate songs, or complex display behaviors, and females choosing among males (or *vice versa* in sex role reversed species).

Today, it has become clear that the underlying cause for the evolution of asymmetric mate choice, at least in species with 'conventional' sex roles (i.e. competitive males and choosy females), is one fundamental feature of sexual organism: Anisogamy and the differential reproductive investment of the sexes (Trivers 1972). Females produce few, large oocytes, while males produce small sperm cells, usually at relatively low costs. Therefore, males can maximize their reproductive success (i.e. the number of offspring they sire) by maximizing the number of matings they attain, while females, in principle, should attempt to optimize mate quality, not quantity, to maximize their reproductive outcome (i.e. the fitness of their offspring) (Bateman 1948, Trivers 1972; see also Andersson 1994; but see e.g. Yasui 1998, Snook 2014 for possible adaptive advantages of polyandry). The often resulting male-biased operational sex ratio (Emlen 1976, Emlen & Oring 1977; see also Clutton-Brock & Parker 1992) forces males to compete for relatively few receptive females and at the same time allows females to choose among relatively many available males (Trivers 1972; see also Kokko et al. 2006).

### 1.1.2 The evolution of secondary sexual characters

Today, numerous models exist for the evolution of male secondary sexual characters. So-called Fisher-Zahavi processes constitute a continuum of sexual selection models that build on the common idea that females gain indirect benefits from choosing among males in the form of genes that enhance their offspring's survival and/or reproductive success (Eshel et al. 2000, Kokko et al. 2002, 2003). On the one end of the continuum, female choice is based in male genetic quality ('good genes model', Zahavi 1975, 1977, Grafen 1990), i.e. on a male's display trait that functions as an 'honest signal' for his genetic quality or constitutes by itself a costly 'handicap'. Thus, female choice for good genes would enhance offspring viability (but not necessarily attractiveness) (Jones et al. 2000, Hine et al. 2002). The other extreme is the so-called Fisherian model of evolution (Fisher 1930, Land 1981). In this case female choice would be arbitrary, i.e. based on a male character that is not necessarily correlated with genetic quality. Since females, thus, increase the attractiveness of their sons (but not necessarily their viability) (Jones et al. 1998), this concept is also referred to as the 'sexy son hypothesis'.

Any variety of the Fisher-Zahavi process may lead to the rapid divergence of secondary sexual characters between populations, especially when females choose for arbitrary traits that are not correlated with male genetic quality (Iwasa & Pomiankowski 1995, Pomiankowski & Iwasa 1998; see also Prum 1997). Yet, also if mate choice is based on good genes, the condition-dependence of honest signals may lead to diverging evolution among populations facing different environmental conditions (Proulx 2001, Reinhold 2004).

Another class of sexual selection models, so-called receiver bias models, assume a male courtship signal to evolve in response to a preexisting female preference (thus, females do not necessarily gain benefits from choosing) (e.g. Endler 1992, Ryan 1998, Ryan & Cummings 2013). Such a preference may either arise as side effect of how the females' sensory system works ('sensory exploitation', e.g. Ryan 1990), or it may have evolved in other contexts, like natural selection for predator avoidance or foraging ('sensory bias', e.g. Christy 1995) (see also Arnqvist 2006). Thus, receiver bias models offer particularly many opportunities for the differentiation of secondary sexual signals among population, for examples due to changes in the females' prey spectrum (e.g. Kolm et al. 2012), but also differential signal transmission in different habitats (for a review of sensory bias in speciation see Boughman 2002).

### 1.1.3 Sexual selection and interspecific diversity

The remarkable diversity of secondary sexual characters, even among closely related taxa, is often attributed to sexual selection promoting rapid evolutionary change and population divergence (e.g. Seehausen & van Alphen 1999, Arnegard et al. 2010, Wagner et al. 2012, Seddon et al. 2013, Bacquet et al. 2015). Support for this hypothesis comes mostly from the often found positive correlation between species richness and various estimates of sexual selection in different taxa (e.g. Barraclough et al. 1995, Arnqvist et al. 2000, Seddon et al. 2008, Kraaijeveld et al. 2011, Wagner et al. 2012; but see e.g. Gage et al. 2002, Morrow et al. 2003, Huang & Rabosky 2014) and the fact that closely related species often differ more dramatically in secondary sexual characters than in other morphological or ecological traits (e.g. Seehausen & van Alphen 1999, Arnegard et al. 2010, Seddon et al. 2013, Simmons 2014, Weber et al. 2016).

Yet, these data are largely correlative or indirect and it has been argued that this does not suffice to conclusively demonstrate the role of sexual selection as a driver of speciation (Panhuis et al. 2001, Ritchie 2007, Kraaijeveld et al. 2011). One of the most serious drawbacks in the study of sexual selection is the fact that its actual impact on the variation of a trait under study cannot commonly be separated from other evolutionary forces potentially contributing to observed trait variation, like natural selection, genetic drift, or mutation (Panhuis et al. 2001, Hosken & House 2011; see also Maan & Seehausen 2011).

## 1.2 Chemical communication and mate choice

### 1.2.1 Pheromones in mate choice

Pheromones are defined as “substances [or substance mixtures] that are secreted by an animal to the outside and cause a specific reaction in a receiving individual of the same species, e.g. a release of certain behavior […]” (Karlson & Butenandt 1959, p. 39; see also Karlson & Lüscher 1959, Wyatt 2010). In the context of sexual communication and mate choice, chemical signals are probably the prevalent means of communication in many animal taxa (Ayasse et al. 2001, Greenfield 2002, Wyatt 2003, Johansson & Jones 2007, Gomez-Diaz & Benton 2013, Steiger & Stökl 2014). Yet, though in recent years a growing number of investigations have dealt with sex pheromones (for reviews see Johansson & Jones 2007, Symonds & Elgar 2008, Smadja & Butlin 2009), research on chemical communication in this context still lags behind, say, the study of

visual and acoustic courtship signals (e.g. Höbel & Gerhardt 2003, Lewis et al. 2004, Ord & Martins 2006, Price et al. 2007, Scholes 2008, Reinhold 2011, Wiley et al. 2011, Blankers et al. 2015; see also Andersson 1994).

In many animal taxa, however, it has been shown that females discriminate among males based on their pheromone (fishes: e.g. Shohet & Watt 2004, Milinski et al. 2005; salamanders: Marco et al. 1998; lizards: e.g. López & Martín 2005, Martín & López 2006; birds: Whittaker et al. 2013; rodents: e.g. Willis & Poulin 2000, Osada et al. 2003; humans: e.g. Thornhill & Gangestad 1999, Chaix et al. 2008). Likewise, in insects, there is evidence for female mate choice based on pheromones as indicators of male quality (e.g. Thornhill 1992, Moore 1994, Iyengar & Eisner 1999a, Iyengar et al. 2001, Jones et al. 2000, Beeler et al. 2002, Hine et al. 2002, Rantala et al. 2002, 2003, Blaul & Ruther 2011, Chemnitz et al. 2015) and in some cases mate discrimination may, at least to some degree, also include choice for Fisherian 'sexy sons' (Jones et al. 1998, Iyengar & Eisner 1999a, 1999b). Moreover, in some species evidence suggests that the male pheromone may exploit a preexisting female sensory bias (Steiger & Stökl 2014), including one of our study species, the European beewolf, *Philanthus triangulum* (Herzner et al. 2005, Schmitt et al. 2007, Steiger et al. 2010; for more details see section 1.4.2.2).

### 1.2.2 The origin of pheromone diversity

The worst decision a female can make in in choosing a mate is deciding for a male from the wrong species. Therefore, species recognition is another integral function of sexual signaling besides intraspecific mate assessment (e.g. Blows & Allan 1998, Klappert & Reinhold 2003, Lewis et al. 2004; see also Andersson 1994, Coyne & Orr 2004). In this regard, pheromones may be a particularly suitable means of communication. Besides quantitative intraspecific variation, qualitative pheromone composition is often remarkably constant within a species, even among geographically isolated populations (Kaltenpoth et al. 2007, Dyer et al. 2014, Knight et al. 2015, Weber et al. 2016), but at the same time markedly different between closely related species (e.g. Symonds & Elgar 2004, Wicker-Thomas 2007, Symonds et al. 2009, Hall et al. 2012, Juárez et al. 2016, Weber et al. 2016). Indeed, pheromone-based avoidance of heterospecific mating has been demonstrated by several studies (Danci et al. 2006, Groot et al. 2006, Dyer et al. 2014, Juárez et al. 2016).

Moreover, the genetics underlying chemical communication in insects can be simple, with often only few genes controlling pheromone production (e.g. Löfstedt 1990, Marcillac et al. 2005,

Tabata & Ishikawa 2005, Domingue et al. 2006, Albre et al. 2012, Niehuis et al. 2013), and genetic studies demonstrate that it needs only relatively small changes in the underlying biosynthetic pathways to fundamentally alter sex pheromone identity and/or composition among closely related taxa (Roelofs et al. 2002, Niehuis et al. 2013, Buček et al. 2015). The same may pertain to the receiver side, as in different insects the response to pheromones seems to be under the control of single or few genes (Hansson et al. 1987, Roelofs et al. 1987, Marcillac et al. 2005, Gould et al. 2010) and even a single amino acid substitution in a chemosensory receptor protein can substantially affect the susceptibility to different pheromone components among populations (Leary et al. 2012). Hence, pheromones are predestined to induce rapid shifts in sexual communication among populations, including the potential for instant reproductive isolation (Symonds & Elgar 2008, Smadja & Butlin 2009).

## 1.3 Exocrine glands in Hymenoptera

### 1.3.1 The morphology of exocrine glands

Insects possess a huge variety of exocrine glands and especially social insects (ants, bees, wasps, and termites), for which up to 150 different exocrine glands are at presence distinguished based on their morphology and location (Billen & Šobotník 2015), have been referred to as “walking glandular batteries” (Billen 1991, p. 67).

By definition, exocrine glands are of ectodermal origin and thus associated with the cuticle (Noirot & Quennedey 1974, Billen 1991). Secretory cells associated with exocrine glands discharge their secretions either directly onto the body surface or into a ductule. The generally accepted classification of insect exocrine glands by Noirot & Quennedey (1974) defines three types of glands (see also Noirot & Quennedey 1991, Quennedey 1998): In class 1 glands, also referred to as epithelial glands (Billen 1991), the secretory cells are in direct contact with the cuticle, thus resembling normal epidermal cells, and the secretion must cross the cuticle through small pores. Class 2 glands have been described as a complex of two cell types, the actual gland cells which do not contact the cuticle, but rather discharge their secretions into surrounding differentiated epidermal cells, so called intercalary cells, from which they cross the cuticle, but are now considered homologous to oenocytes (Billen 1991, Noirot & Quennedey 1991, Quennedey 1998). Class 3 glands, are made up by bicellular secretory units consisting of the actual gland cell and an associated canal cell that is associated with the gland cell through the so-called end apparatus and

transfers the secretion to the cuticle via a ductule. Sometimes, several class 3 gland cells are tightly clustered, forming so-called 'acini', which are jointly connected to the cuticle by bundles of conducting canals (e.g. Goettler & Strohm 2008).

### 1.3.2 Exocrine glands and antimicrobial defense

As diverse as is the morphology of exocrine glands, are the functions their secretions serve in Hymenoptera, as in other insects, including digestion (e.g. Cruz-Landim & Costa 1998, do Amaral & Caetano 2005), the production of comb wax (e.g. Hepburn et al. 2014) and silk (Fisher & Robertson 1999, Hölldobler et al. 2014), predator deterrence (e.g. Laurent et al. 2005, Stökl & Herzner 2016), and even the cultivation and sustenance of protective symbiotic bacteria (Kaltenpoth et al. 2005, Currie et al. 2006, Goettler et al. 2007). Moreover, that exocrine gland secretions themselves can act as antimicrobials has been demonstrated for a number of solitary and social Hymenoptera (solitary wasps: Willers et al. 1982, Führer & Willers 1986; solitary bees: Cane et al. 1983; ants: reviewed in Tragust 2016) and other insects (Flies: e.g. Jaklič et al. 2008, Pöppel et al. 2014; beetles: e.g. Gross et al. 1998, 2002, 2008, Prendeville & Stevens 2002, Rozen et al. 2008, Pedrini et al. 2015; termites: Bulmer et al. 2009, Hamilton et al. 2011; earwigs: Gasch et al. 2013).

Antimicrobial secretions are employed by adult insects to disinfect their own body surface, and also their environment including food and nest substrate, as well as their brood and nestmates (Otti et al. 2014). Yet, also larval secretions can serve as antimicrobials, as has for instance been demonstrated for larvae of the parasitoid wasp *Ampulex compressa* (Hymenoptera, Ampulicidae), which disinfect their hosts, American cockroaches, and the surrounding brood cell with antimicrobial oral secretions before they spin their cocoon inside the host carcass (Herzner et al. 2013a, Weiss et al. 2014).

An explicitly fascinating example of antimicrobial brood defense has been described for our focal taxon, the Philanthinae. In some species, females possess large and complex postpharyngeal glands (PPGs) (Strohm et al. 2007, Herzner et al. 2013b; chapter 2). They apply high amounts of the lipid secretion from their large PPGs to the cuticle of their prey, paralyzed bees (Herzner & Strohm 2007, 2008, Herzner et al. 2007a, 2013b; chapter 2). This secretion alters the physicochemical properties of the prey's cuticle and reduces the condensation of water, which renders fungal growth (Herzner & Strohm 2007; for more details see section 1.4.2.1).

### 1.3.3 Exocrine glands and sexual communication

In Hymenoptera, exocrine glands moreover play important roles in intraspecific communication, including alarm and recruitment (e.g. Blum 1969, Wilson & Regnier 1971, Crewe et al. 1972, Billen & Morgan 1998), swarm formation (Winston et al. 1998), nestmate (reviewed in Lenoir et al. 1999) and queen recognition (Slessor et al. 1988, Winston & Slessor 1992), trail laying (Billen & Morgan 1998, Billen 2009), host marking (Godfrey 1994, Quicke 1997, Anderson 2002, Stökl & Herzner 2016), and sexual signaling (e.g. Ayasse et al. 2001). For recent reviews of the diverse functions of exocrine glands in pheromone communication in the honey bee (*Apis mellifera*) and ants see also Slessor et al. (2005) and Morgan (2008), respectively.

One gland that is often involved in sexual communication in both male and female Hymenoptera is the mandibular gland (MG) (e.g. Ayasse et al. 2001). It is common to all Hymenoptera (bees: e.g. Cruz-Landim 1967, Cruz-Landim et al. 2005, Galvani & Settembrini 2013; apoid wasps: Duffield et al. 1981, Penagos-Arévalo et a. 2015; vespid wasps: Pietrobon & Caetano 2003; ants: e.g. Blum 1969, do Amaral & Machado-Santelli 2008; parasitoid wasps: Stökl & Herzner 2016, Zimmermann & Vilhelmsen 2016) and generally consists of paired reservoirs associated with class 3 gland cells that open at the base of the mandibles (e.g. Cruz-Landim & Reginato 2001, Grasso et al. 2004, Goettler & Strohm 2008, Billen et al. 2013, Stökl & Herzner 2016, Zimmermann & Vilhelmsen 2016). The MG has been found to produce the male territory marking secretion in *Centris* (Vinson et al. 1982, 1984) and *Xylocopa* bees (Hefetz 1983), as well as *Polistes* wasps (Wenzel 1987), and the female sex pheromone in *Leptopilina* parasitoids (Stökl & Herzner 2016).

In the Philanthinae, an MG is present in both sexes and its morphology has been studied in males of the European beewolf *P. triangulum* (Goettler & Strohm 2008) and both sexes of *Cerceris rybyensis* (Ågren 1977). In male *P. triangulum* the MG is of enormous size and associated with a large number of acini of class 3 gland cells (Goettler & Strohm 2008). Its likely function is the production of a marking pheromone (Goettler & Strohm 2008) that male Philanthinae use to attract receptive females to their territory (Alcock 1975, Evans & O'Neill 1985, 1988, Schmidt et al. 1985, Clarke et al. 2001, O'Neill 2001, Schmitt et al. 2003, Kroiss et al. 2006). Yet, in *P. triangulum*, the PPG is likely the actual storage organ for the high amounts of pheromone produced in the gland cells of the MG (Kroiss et al. 2006, Herzner et al. 2007b).

## 1.4 The Philanthinae

### 1.4.1 Systematics and geographic distribution

The Philanthinae constitute one of the largest subfamilies of the digger wasp family Crabronidae, comprising well over 1,100 described species with a nearly worldwide distributions (excluding Antarctica) (Bohart & Menke 1976, Pulawski 2017). The eight genera of the Philanthinae are separated into three tribes (Figure 1.1, Alexander 1992). The largest tribe, the Cercerini, in total including over 900 species (Pulawski 2017), comprise (*Cerceris* + *Eucerceris*) + *Pseudoscolia* (though the later genus is often assigned to a separate tribe, the Pseudoscoliini; e.g. Bohart & Menke 1976, Pulawski 2017). The Aphilanthopsini, on the other hand, comprise only 13 species from the two genera *Clypeadon* and *Aphilanthops* (Pulawski 2017). Finally, the Philanthini, the so-called beewolves, represent about 170 species (Pulawski 2017) and comprise (*Philanthus* + *Trachypus*) + *Philanthinus* (with *Trachypus* most probably forming a monophyletic clade within paraphyletic *Philanthus* rather than a proper genus; Alexander 1992, Kaltenpoth et al. 2014).

Throughout the work presented in this thesis, the main focus lies on the tribe Philanthini. According to a molecular phylogeny of Kaltenpoth et al. (2014), the three taxa of the Philanthini can be grouped into five phylogenetic clades that largely coincide with their geographic distribution (Figure 1.1): (1) The small Palearctic genus *Philanthinus* constituting the very basal taxon of the tribe, (2) a small clade of Palearctic, Indian, and Afrotropical species of the genus *Philanthus*, forming the sister group to all other *Philanthus*, (3) a clade comprising all other Palearctic, Indian, and Afrotropical *Philanthus*, (4) all Nearctic *Philanthus*, and (5) the clade comprising the Neotropical *Trachypus*.

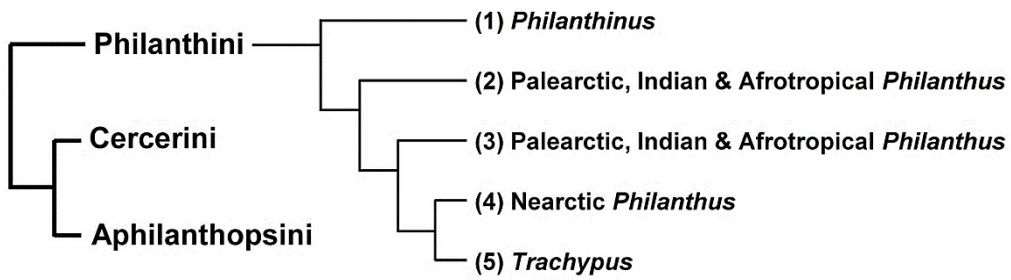


**Figure 1.1** Phylogenetic relationships among the three tribes of the Philanthinae, as well as the different phylogenetic clades within the Philanthini according to Kaltenpoth et al. (2014).

### 1.4.2 General biology

Most literature concerning the biology of the Philanthinae focuses on the genera *Philanthus* and *Cerceris*, with the best investigated species certainly being the European beewolf *P. triangulum* (Tinbergen 1932, 1935, Rathmayer 1962, Simon-Thomas & Poorter 1972, Simon-Thomas & Veenendaal 1978, Strohm 1995, 2000, Strohm & Linsenmair 1994/1995, 1997, 1999, 2000, 2001, Strohm & Lechner 2000, Strohm & Marliani 2002). All members of the subfamily Philanthinae share basic life-history characters, in particular with regard to male and female reproductive behavior (e.g. Bohart & Menke 1976, Evans & O'Neill 1988). The following sections summarize our current knowledge of the biology and behavior of beewolves, as well as the other philanthine tribes Cercerini and Aphilanthopsini.

#### *1.4.2.1 Female nesting behavior*

Female Philanthinae built subterranean nests in sandy, sun-exposed soil in which brood cells are successively constructed for each single larva (for detailed descriptions of nest architecture of various species see e.g. Evans 1962, 1971, 2000, Evans & Matthews 1973, Evans & O'Neill 1988, Giovanetti 2005). Brood cells are mass-provisioned with paralyzed arthropod prey as larval food (Evans 1962, 1971, 2000, Bohart & Menke 1976, Evans & O'Neill 1988, Polidori et al. 2005, 2006) and after laying a single egg on one of the stored prey items, the female closely seals the brood cell, providing no further care to its offspring (e.g. Strohm & Linsenmair 1994/95).

Regarding the prey spectrum used, there are marked differences between the tribes. All species of the Philanthini provision their brood with different, mostly aculeate Hymenoptera, predominantly bees and occasionally wasps (e.g. Evans & Matthews 1973, Evans & O'Neill 1988, Wcislo et al. 2004, Koedam et al. 2009, 2011). Many Philanthini have a rather broad prey spectrum, often comprising species from different Hymenopteran families (e.g. Bohart & Menke 1976, Evans & O'Neill 1988), while some species are specialized on one prey species, like, for instance, the European beewolf *P. triangulum* and the Indian *Philanthus basalis*, which are specialized predators of workers of the European honeybee, *A. mellifera* (e.g. Strohm & Linsenmair 1997, Strohm & Marliani 2002), and the Asian honeybee, *Apis cerana indica* (Krombein 1981), respectively, or the South American *Trachypus boharti*, solely hunting for males of the stingless bee *Scaptotrigona postica* (Koedam et al. 2009, 2011).

As far as is known, all Aphilanthopsini hunt exclusively on ants (Wheeler 1913, Ristich 1956, Evans 1962, Alexander 1986). Most members of the Cercerini rely on Coleoptera (mainly weevils and buprestids) as larval provisions (e.g. Evans 1971, Bohart & Menke 1976, Giovanetti 2005, Polidori et al. 2005, 2006, 2007, 2010), while in the two *Pseudoscolia* species for which prey records are known, females hunt for halictid bees (Bohart & Menke 1976, Asis et al. 1991). Likewise, some species of the genus *Cerceris* prey upon bees, especially Halictidae (e.g. Bohart & Menke 1976, Polidori et al. 2005, 2006, 2007, 2010), thus resembling the beewolves of the tribe Philanthini.

Due to the warm and humid microclimate of the brood cell and the presence of potentially pathogenic mold fungi in the surrounding soil (Engl et al. 2016), larval provisions are under a high risk of being infested by competing or detrimental microorganisms (e.g. Strohm & Linsenmair 2001) that can severely affect larval fitness and survival (Herzner et al. 2011). Yet, in *P. triangulum* only 5.3% of brood cells have been found to be actually infested by mold under natural conditions (Strohm & Linsenmair 2001). This rather low infection level is assigned to the evolution of different effective counter strategy, at least in some species of the Philanthini.

In *P. triangulum*, as well as the two closely related species *Philanthus gibbosus* and *Trachypus elongatus*, females possess large PPGs (Strohm et al. 2007, Herzner et al. 2013b; chapter 2). By extensively licking the surface of their paralyzed prey, females applying high amounts of the lipid secretion from their PPGs to the prey's cuticle, a behavior that has been termed 'prey embalming' (Herzner & Strohm 2007, 2008, Herzner et al. 2007a, 2013b; chapter 2). The secretion of the PPG largely consists of unsaturated long-chain hydrocarbons (HCs) (Herzner et al. 2007a, 2013b, Strohm et al. 2008; chapter 2). Prey embalming, therefore, alters the physicochemical properties of the prey's cuticle and reduces the condensation of water, which renders the microclimate on the paralyzed prey less suitable for fungal growth (Herzner & Strohm 2007). Thus, prey embalming retards the decomposition of the stored prey and in *P. triangulum* considerably increases larval survival (Strohm & Linsenmair 2001, Herzner et al. 2011).

Another antimicrobial strategy of the Philanthini is the symbiosis with antibiotic-producing *Streptomyces* bacteria (Kaltenpoth et al. 2006). In all three genera of the Philanthini, *Philanthus*, *Trachypus*, and *Philanthinus*, these symbionts are harbored in specialized antennal glands of the females (Kaltenpoth et al. 2006, 2010, 2012, 2014). Females secret the bacteria into the brood cell (Kaltenpoth et al. 2005, Goettler et al. 2007) where they are incorporated into the cocoon by the larva and protect it from detrimental microorganisms during hibernation (Kaltenpoth et al. 2005,

Kroiss et al. 2010a, Koehler et al. 2013). Thus far, this antimicrobial strategy has not been detected in any species of the Cercerini and Aphilanthopsini (Kaltenpoth et al. 2006).

*1.4.2.2 Male territorial behavior*

As far as is known, males of all but one species of the Philanthinae establish small territories that often aggregate in the vicinity of female nesting sites (Alcock 1975, Gwynne 1978, Steiner 1978, Evans & O'Neill 1985, 1988, Strohm 1995, Strohm & Lechner 2000, Clarke et al. 2001, O'Neill 2001, Kroiss et al. 2010b). The only known species in which males are not territorial is the North American *Philanthus albopilosus* (Evans & O'Neill 1988). To establish territories, males mark plant stems or blades with a secretion from their head glands (Alcock 1975, Schmidt et al. 1985, Evans & O'Neill 1988, Schmitt et al. 2003, Kroiss et al. 2006). By running over the plant surface, males apply these marking secretions to the surface with their clypeal brushes thereby conspicuously bending both their head and abdomen against the surface (e.g. Evans & O'Neill 1988). The territories are defended against conspecific males and sometimes other intruders (Evans & O'Neill 1988, Kroiss et al. 2010b; for a detailed description of male territorial behavior in *P. triangulum* see e.g. Simon-Thomas & Poorter 1972). Territories seem not to contain any resources attractive to females, like flowers for nectar feeding or hunting (Kroiss et al. 2010b). Females approach territories in a zig-zagging flight from downwind, indicating that they are indeed attracted by and orientate towards the male markings (e.g. Evans & O'Neill 1988). When a female alights in a territory mating is initiated immediately, apparently without further male display (Evans & O'Neill 1988, Kroiss et al. 2010b).

Detailed information of male territorial marking and defense behavior, however, is largely restricted to the genus *Philanthus*. Only for the European beewolf *P. triangulum*, both the marking secretion (Schmidt et al. 1990, Schmitt et al. 2003, Herzner et al. 2007b) as well as the glands involved in its production (Herzner et al. 2007b, Goettler & Strohm 2008) have recently been examined in detail. Male *P. triangulum* disperse vast amounts of marking secretion (Schmitt et al. 2003, Kroiss et al. 2006) the main compound of which is (*Z*)-11-eicosen-1-ol (Schmidt et al. 1990, Schmitt et al. 2003, Herzner et al. 2007b), which has likely evolved due to a female sensory bias (Herzner et al. 2005, Schmitt et al. 2007, Steiger et al. 2010; for more details see section 6.2.3 in chapter 6). Few publications exist on the chemistry of the marking secretions in some other *Philanthus* species (Gwynne 1978, McDaniel et al. 1987, 1992, Schmidt et al. 1985, 1990), as well as *Eucerceris* (Clark et al. 2001), and besides *P. triangulum*, the respective glands have thus far only been investigated in *Cerceris rybyensis* (Ågren 1977).

In *Philanthus*, the general close association of male territories in leks (Evans & O'Neill 1988, O'Neill 2001, Kroiss et al. 2010b) probably allows females to directly compare males and facilitates mate choice. In *Philanthus*, and likely also other male Philanthinae, the territorial markings seem the sole means of males to attract receptive females, and at the same time may provide females with a wealth of potentially information on male quality (Kaltenpoth & Strohm 2006, Herzner et al. 2006, Kaltenpoth et al. 2007, Roeser-Mueller et al. 2010). Thus the male marking pheromone is likely crucial for both males and females to maximize their fitness returns.

## 1.5 Outline of the thesis

In the present thesis, we study the interspecific variation in the exocrine head glands of beewolves and related taxa. In the following chapters (chapters 2-4), we assess the amount of variation in head gland morphology, as well as the chemical composition of PPG secretion among female Philanthinae and its implications for the evolution of the unique antimicrobial prey embalming behavior described for *P. triangulum* and *T. elongatus* (Strohm and Linsenmair 2001, Herzner et al. 2011, 2013b). In chapter 2 we present evidence that females of the North American *P. gibbosus* likewise employ prey embalming, corroborating the supposed ubiquity of this antimicrobial strategy within the Philanthini. Furthermore, we show that, in accordance with this hypothesis, female beewolves possess uniformly large and complex PPGs that strongly differ from the PPGs of the related tribes Cercerini and Aphilanthopsini, which are simple, tube-shaped evaginations. The comparison of the chemical composition of the PPG content among female Philanthinae (chapter 3) points in the same direction, with the PPG of all female beewolves being by far dominated by unsaturated long-chain HCs, while the investigated members of the other tribes show some variation in their PPG chemistry. Since one possible trigger for the evolution of the costly prey embalming mechanism in the Philanthini may be the use of bees as prey (see discussion in chapter 2), chapter 4 more closely investigates the PPG morphology in the genus *Cerceris*. Since in this genus some species hunt on bees, while most species rely on beetles as larval provisions, possible differences in PPG morphology among bee- and beetle-hunting *Cerceris* would have allowed conclusions about the role of prey use in the evolution of embalming. Yet, we found no differences in PPG morphology between bee- and beetle-hunting species.

Chapter 5 deals with the evolution of interspecific variation in the head glands of male Philanthinae. Male Philanthinae use marking pheromones to attract receptive females (e.g. Evans & O'Neill 1988), which, in *P. triangulum* and most likely all other Philanthinae, is produced and

stored in the MG and/or PPG (Kroiss et al. 2006, Herzner et al. 2007b, Goettler & Strohm 2008). Thus, as opposed to females, male exocrine glands are likely under strong sexual selection and, as has often been hypothesized for sexually selected traits (e.g. Seehausen & van Alphen 1999, Arnegard et al. 2010, Wagner et al. 2012, Seddon et al. 2013, Bacquet et al. 2015), should therefore show higher interspecific variation as compared to females. To approach this hypothesis, we conducted a comparative multivariate analysis of the morphology of the presumptive pheromone glands among male Philanthinae, which revealed an astonishing degree of interspecific variation, including losses and regains of entire glands and the occurrence of novel types of gland cells, yet following a strong overall phylogenetic trend. We then conducted a joint multivariate statistical analysis of male and female head gland morphology, to assess the contribution of sexual selection to interspecific variation in male glands against the females' glands as a 'reference under natural selection'. In chapter 6, I conclude with a general discussion of different aspects of the evolution of exocrine head glands in the Philanthinae, including the possible origin of the unique antimicrobial brood protection mechanism involving females' PPG secretion, as well as the potential selective forces shaping the complex evolutionary pattern in male pheromone glands, especially in the context of preliminary results on male pheromone chemistry, and the possible concerted evolution of male and female gland morphology due to genetic constrains.

## 1.5 References

Ågren L: Mandibular gland morphology of *Cerceris rybyensis* (L) (Hymenoptera: Philanthidae). *Zoon* 1977, 5(2):91-95.

Albre J, Liénard MA, Sirey TM, Schmidt S, Tooman LK, Carraher C, Greenwood DR, Löfstedt C, Newcomb RD: Sex pheromone evolution is associated with differential regulation of the same desaturase gene in two genera of leafroller moths. *Plos Genetics* 2012, 8(1).

Alcock J: Male mating strategies of some philanthine wasps (Hymenoptera: Sphecidae). *Journal of the Kansas Entomological Society* 1975, 48(4):532-545.

Alexander BA: Alternative methods of nest provisioning in the digger wasp *Clypeadon laticinctus* (Hymenoptera: Sphecidae). *Journal of the Kansas Entomological Society* 1986, 59-63.

Alexander BA: A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 1992, 17(2):91-108.

Anderson P: Oviposition pheromones in herbivorous and carnivorous insects. In: Chemical ecology of insect eggs and egg deposition. Edited by Hilker M, Meiners T. Berlin: Blackwell Publishing, 2002.

Andersson MB: Sexual selection: Princeton University Press, 1994.

Arnegard ME, McIntyre PB, Harmon LJ, Zelditch ML, Crampton WGR, Davis JK, Sullivan JP, Lavoue S, Hopkins CD. Sexual signal evolution outpaces ecological divergence during electric fish species radiation. *The American Naturalist* 2010, 176(3):335-356.

Arnqvist G: Sensory exploitation and sexual conflict. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 2006, 361(1466):375-386.

Arnqvist G, Edvardsson M, Friberg U, Nilsson T: Sexual conflict promotes speciation in insects. *Proceedings of the National Academy of Sciences* 2000, 97(19):10460-10464.

Asis J, Gayubo S, Tormos J: Description of Mature Larva and Nesting Behavior of *Pseudoscolia martinezi* Suárez (Hymenoptera: Sphecidae). *Journal of the New York Entomological Society* 1991:261-266.

Ayasse M, Paxton R, Tengö J: Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology* 2001, 46(1):31-78.

Bacquet PMB, Brattström O, Wang HL, Allen CE, Löfstedt C, Brakefield PM, Nieberding CM: Selection on male sex pheromone composition contributes to butterfly reproductive isolation. *Proceedings of the Royal Society B: Biological Sciences* 2015, 282(1804):20142734.

Barraclough TG, Harvey PH, Nee S: Sexual selection and taxonomic diversity in passerine birds. *Proceedings of the Royal Society of London B: Biological Sciences* 1995, 259(1355):211-215.

Bateman AJ: Intra-sexual selection in *Drosophila*. *Heredity* 1948, 2(3):349-368.

Beeler AE, Rauter CM, Moore AJ: Mate discrimination by females in the burying beetle *Nicrophorus orbicollis*: The influence of male size on attractiveness to females. *Ecological Entomology* 2002, 27(1), 1-6.

Billen J: Ultrastructural Organization of the Exocrine Glands in Ants. *Ethology Ecology & Evolution* 1991, 1:67-73.

Billen J: Occurrence and structural organization of the exocrine glands in the legs of ants. *Arthropod Structure & Development* 2009, 38(1):2-15.

Billen J, Morgan ED: Pheromone communication in social insects: sources and secretions. In: Pheromone communication in social insects. Edited by Vander Meer RK, Breed MD, Espelie KE, Winston ML: Westview Press, 1998: 3-33.

Billen J, Šobotník J: Insect exocrine glands. *Arthropod Structure & Development* 2015, 44:399-400.

Billen J, Bauweleers E, Hashim R, Ito F: Survey of the exocrine system in *Protanilla wallacei* (Hymenoptera, Formicidae). *Arthropod Structure & Development* 2013, 42(3):173-183.

Blankers T, Lübke A, Hennig R: Phenotypic variation and covariation indicate high evolvability of acoustic communication in crickets. *Journal of Evolutionary Biology* 2015, 28(9):1656-1669.

Blaul B, Ruther J: How parasitoid females produce sexy sons: a causal link between oviposition preference, dietary lipids and mate choice in *Nasonia*. *Proceedings of the Royal Society of London B: Biological Sciences* 2011, rspb20110001.

Blows MW, Allan RA: Levels of mate recognition within and between two *Drosophila* species and their hybrids. *The American Naturalist* 1998, 152(6):826-837.

Blum MS: Alarm Pheromones. *Annual Review of Entomology* 1969, 14:57-80.

Bohart RM, Menke AS: Sphecid wasps of the world: a generic revision. 1st ed. Ithaca: University of California Press, 1976.

Boughman JW: How sensory drive can promote speciation. *Trends in Ecology & Evolution* 2002, 17(12):571-577.

Buček A, Matoušková P, Vogel H, Šebesta P, Jahn U, Weißflog J, Svatoš A, Pichová I: Evolution of moth sex pheromone composition by a single amino acid substitution in a fatty acid desaturase. *Proceedings of the National Academy of Sciences* 2015, 112(41):12586-12591.

Bulmer MS, Bachelet I, Raman R, Rosengaus RB, Sasisekharan R: Targeting an antimicrobial effector function in insect immunity as a pest control strategy. *Proceedings of the National Academy of Sciences of the United States* 2009, 106:12652–12657.

Cane JH, Gerdin S, Wife G: Mandibular gland secretions of solitary bees (Hymenoptera, Apoidea) - Potential for nest cell disinfection. *Journal of the Kansas Entomological Society* 1983, 56(2):199-204.

Chaix R, Cao C, Donnelly P: Is mate choice in humans MHC-dependent? *PLoS Genetics* 2008, 4(9), e1000184.

Chemnitz J, Jentschke PC, Ayasse M, Steiger S: Beyond species recognition: somatic state affects long-distance sex pheromone communication. *Proceedings of the Royal Society of London B: Biological Sciences* 2015, 282(1812):20150832.

Christy JH: Mimicry, mate choice, and the sensory trap hypothesis. *The American Naturalist* 1995, 146(2):171-181.

Clarke S, Dani F, Jones G, Morgan E, Schmidt J: (Z)-3-hexenyl (R)-3-hydroxybutanoate: a male specific compound in three North American decorator wasps *Eucerceris rubripes*, *E. conata* and *E. tricolor*. *Journal of Chemical Ecology* 2001, 27(7):1437-1447.

Clutton-Brock TH, Parker GA: Potential reproductive rates and the operation of sexual selection. *The Quarterly Review of Biology* 1992, 67(4):437-456.

Coyne J, Orr H: Speciation: Sinauer Associates Inc., 2004.

Crewe RM, Blum MS, Collingwood CA: Comparative analysis of alarm pheromones in the ant genus *Crematogaster*. *Comparative Biochemistry and Physiology B* 1972, 43(3):703-716.

Cruz-Landim C: Estudo comparativo de algumas glândulas das abelhas (Hymenoptera, Apoidea) e respectivas implicacoes evolutivas. *Arquivos de Zoologia, Sao Paulo* 1967, 15(3):177-290.

Cruz-Landim C, Costa R: Structure and function of the hypopharyngeal glands of Hymenoptera: A comparative approach. *Journal of Comparative Biology* 1998, 3(2):151-163.

Cruz-Landim C, Reginato RD: Exocrine glands of *Schwarziana quadripunctata* (Hymenoptera, Apinae, Meliponini). *Brazilian Journal of Biology* 2001, 61(3):497-505.

Cruz-Landim C, Abdalla FC, Gracioli-Vitti LF: Morphological and functional aspects of volatile-producing glands in bees (Hymenoptera: Apidae). *Insect Sciences* 2005, 12(6):467-480.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J: Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 2006, 311(5757):81-83.

Danci A, Schaefer PW, Schopf A, Gries G: Species-specific close-range sexual communication systems prevent cross-attraction in three species of *Glyptapanteles* parasitic wasps (Hymenoptera: Braconidae). *Biological Control* 2006, 39(2):225-231.

Darwin C: On the origin of species by means of natural selection. 1st ed. London: John Murray, 1859.

Darwin C: The descent of man and selection in relation to sex. 2nd ed. London: John Murray, 1871.

do Amaral JB, Caetano FH: The hypopharyngeal gland of leaf-cutting ants (*Atta sexdens rubropilosa*) (Hymenoptera : Formicidae). *Sociobiology* 2005, 46(3):515-524.

do Amaral JB, Machado-Santelli G: Salivary system in leaf-cutting ants (*Atta sexdens rubropilosa* Forel, 1908) castes: A confocal study. *Micron* 2008, 39(8):1222-1227.

Domingue MJ, Starmer WT, Teale SA: Genetic control of the enantiomeric composition of ipsdienol in the pine engraver, *Ips pini*. *Journal of Chemical Ecology* 2006, 32(5):1005.

Duffield RM, Shamim M, Wheeler JW, Menke AS: Alkylpyrazines in the mandibular gland secretions of *Ammophila* wasps (Hymenoptera: Sphecidae). *Comparative Biochemistry and Physiology B* 1981, 70(2):317-318.

Dyer KA, White BE, Sztepanacz JL, Bewick ER, Rundle HD: Reproductive character displacement of epicuticular compounds and their contribution to mate choice in *Drosophila subquinaria* and *Drosophila recens*. *Evolution* 2014, 68(4):1163-1175.

Emlen ST: Lek organization and mating strategies in the bullfrog. *Behavioral Ecology and Sociobiology* 1976, 1(3):283-313.

Emlen ST, Oring LW: Ecology, sexual selection, and the evolution of mating systems. *Science* 1977, 197(4300):215-223.

Endler JA: Signals, signal conditions, and the direction of evolution. *The American Naturalist* 1992, 139:S125-S153.

Engl T, Bodenstein B, Strohm E: Mycobiota in the brood cells of the European beewolf, *Philanthus triangulum* (Hymenoptera: Crabronidae). *European Journal of Entomology* 2016, 113(1):271-277.

Eshel I, Volovik I, Sansone E: On Fisher-Zahavi's handicapped sexy son. *Evolutionary Ecology Research* 2000, 2:509-523.

Evans HE: A review of nesting behavior of digger wasps of the genus *Aphilanthops*, with special attention to the mechanism of prey carriage. *Behaviour* 1962, 19:239-260.

Evans HE: Observations on the nesting behavior of wasps of the tribe Cercerini. *Journal of the Kansas Entomological Society* 1971, 44(4):500-523.

Evans HE: Observations on the biology of *Cerceris mimica* Cresson (Hymenoptera: Sphecidae: Philanthinae). *Journal of the Kansas Entomological Society* 2000, 73(4):220-224.

Evans HE, O'Neill KM. Male territorial behavior in four species of the tribe Cercerini (Sphecidae: Philanthinae). *Journal of the New York Entomological Society* 1985, 93(3):1033-1040

Evans HE, O'Neill KM: The natural history and behavior of North American beewolves. 1st ed. Ithaca: Cornell University Press, 1988.

Evans HE, Matthews RW: Observations on the nesting behavior of *Trachypus petiolatus* (Spinola) in Colombia and Argentina (Hymenoptera: Sphecidae: Philanthini). *Journal of the Kansas Entomological Society* 1973:165-175.

Fisher RA: The genetical theory of natural selection. Oxford University Press, 1930.

Fisher B, Robertson H: Silk production by adult workers of the ant *Melissotarsus emeryi* (Hymenoptera, Formicidae) in South African fynbos. *Insectes Sociaux* 1999, 46(1):78-83.

Führer E, Willers D: The anal secretions of the endoparasitic larva *Pimpla turionellae*: Sites of production and effects. *Journal of Insect Physiology* 1986, 32:361-367.

Gage MJ, Parker GA, Nylin S, Wiklund C: Sexual selection and speciation in mammals, butterflies and spiders. *Proceedings of the Royal Society of London B: Biological Sciences* 2002, 269(1507):2309-2316.

Galvani GL, Settembrini BP: Comparative morphology of the head glands in species of Protepeolini and Emphorini (Hymenoptera: Apidae). *Apidologie* 2013, 44(4):367-381.

Gasch T, Schott M, Wehrenfennig C, Düring RA, Vilcinskas A: Multifunctional weaponry: the chemical defenses of earwigs. *Journal of Insect Physiology* 2013, 59(12):1186-1193.

Giovanetti M: Nesting ecology of a neotropical solitary wasp (Hymenoptera: Sphecidae) in Panamá. *Neotropical Entomology* 2005, 34(5):713-719.

Godfray HCJ: Parasitoids: Behavioral and evolutionary ecology. Princton: Princton University Press, 1994.

Goettler W, Strohm E: Mandibular glands of male European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2008, 37(5):363-371.

Goettler W, Kaltenpoth M, Herzner G, Strohm E: Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36(1):1-9.

Gomez-Diaz C, Benton R: The joy of sex pheromones. *EMBO Reports* 2013, 14(10):874-883.

Gould F, Estock M, Hillier NK, Powell B, Groot AT, Ward CM, Emerson JL, Schal C, Vickers NJ: Sexual isolation of male moths explained by a single pheromone response QTL containing four receptor genes. *Proceedings of the National Academy of Sciences* 2010, 107(19):8660-8665.

Grafen A: Biological signals as handicaps. *Journal of theoretical Biology* 1990, 144(4):517-546.

Grasso D, Romani R, Castracani C, Visicchio R, Mori A, Isidoro N, Le Moli F: Mandible associated glands in queens of the slave-making ant *Polyergus rufescens* (Hymenoptera, Formicidae). *Insectes Sociaux* 2004, 51(1):74-80.

Greenfield MD: Signalers and receivers: Mechanisms and evolution of arthropod communication. Oxford: Oxford University Press, 2002.

Groot AT, Horovitz JL, Hamilton J, Santangelo RG, Schal C, Gould F: Experimental evidence for interspecific directional selection on moth pheromone communication. *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103(15):5858-5863.

Gross J, Müller C, Vilcinskas A, Hilker M: Antimicrobial activity of exocrine glandular secretions, hemolymph, and larval regurgitate of the mustard leaf beetle *Phaedon cochleariae*. *Journal of Invertebrate Pathology* 1998, 72(3):296-303.

Gross J, Podsiadlowski L, Hilker M: Antimicrobial activity of exocrine glandular secretion of *Chrysomela* larvae. *Journal of Chemical Ecology* 2002, 28(2):317-331.

Gross J, Schumacher K, Schmidtberg H, Vilcinskas A: Protected by fumigants: Beetle perfumes in antimicrobial defense. *Journal of Chemical Ecology* 2008, 34(2):179-188.

Gwynne DT: Male territoriality in bumblebee wolf, *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae) - Observations on behavior of individual males. *Zeitschrift für Tierpsychologie* 1978, 47(1):89-103.

Hall DR, Amarawardana L, Cross JV, Francke W, Boddum T, Hillbur Y: The chemical ecology of cecidomyiid midges (Diptera: Cecidomyiidae). *Journal of Chemical Ecology* 2012, 38(1):2-22.

Hamilton C, Lay F, Bulmer MS: Subterranean termite prophylactic secretions and external antifungal defenses. *Journal of Insect Physiology* 2011, 57:1259–1266.

Hansson B, Löfstedt C, Roelofs W: Inheritance of olfactory response to sex pheromone components in *Ostrinia nubilalis*. *Naturwissenschaften* 1987, 74(10):497-499.

Hefetz A: Function of secretion of mandibular gland of male in territorial behavior of *Xylocopa sulcatipes* (Hymenoptera, Anthophoridae). *Journal of Chemical Ecology* 1983, 9(7):923-931.

Hepburn H, Pirk C, Duangphakdee O: The Wax Gland Complex. In: Honeybee Nests: Composition, Structure, Function. Edited by Hepburn H, Pirk C, Duangphakdee O. Berlin: Springer, 2014: 301-318.

Herzner G, Strohm E: Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007, 17(2):R46-R47.

Herzner G, Strohm E: Food wrapping by females of the European Beewolf, *Philanthus triangulum*, retards water loss of larval provisions. *Physiological Entomology* 2008, 33(2):101-109.

Herzner G, Schmitt T, Linsenmair KE, Strohm E: Prey recognition by females of the European beewolf and its potential for a sensory trap. *Animal Behaviour* 2005, 70(6):1411-1418.

Herzner G, Schmitt T, Heckel F, Schreier P, Strohm E: Brothers smell similar: variation in the sex pheromone of male European Beewolves *Philanthus triangulum* F. (Hymenoptera : Crabronidae) and its implications for inbreeding avoidance. *Biological Journal of the Linnean Society* 2006, 89(3):433-442.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007a, 33(4):849-859.

Herzner G, Goettler W, Kroiss J, Purea A, Webb AG, Jakob PM, Roessler W, Strohm E: Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 2007b, 36(2):123-133.

Herzner G, Engl T, Strohm E: Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011, 82(2):321-328.

Herzner G, Schlecht A, Dollhofer V, Parzefall C, Harrar K, Kreuzer A, Pilsl L, Ruther J: Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials. *Proceedings of the National Academy of Science of the United States* 2013a, 110.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, Chemistry and Function of the Postpharyngeal Gland in the South American Digger Wasps *Trachypus boharti* and *Trachypus elongatus*. *Plos One* 2013b, 8(12).

Hine E, Lachish S, Higgie M, Blows MW: Positive genetic correlation between female preference and offspring fitness. *Proceedings of the Royal Society of London B: Biological Sciences* 2002, 269(1506):2215-2219.

Höbel G, Gerhardt HC: Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). *Evolution* 2003, 57(4), 894-904.

Hölldobler B, Obermayer M, Plowes NJ, Fisher BL: New exocrine glands in ants: the hypostomal gland and basitarsal gland in the genus *Melissotarsus* (Hymenoptera: Formicidae). *Naturwissenschaften* 2014, 101(7):527-532.

Hosken DJ, House CM: Sexual selection. *Current Biology* 2011, 21(2):R62-R65.

Huang H, Rabosky DL: Sexual selection and diversification: Reexamining the correlation between dichromatism and speciation rate in birds. *The American Naturalist* 2014, 184(5):E101-E114.

Huxley JS: Darwin's theory of sexual selection and the data subsumed by it, in the light of recent research. *The American Naturalist* 1938, 72(742):416-433.

Iwasa Y, Pomiankowski A: Continual change in mate preferences. *Nature* 1995, 377(6548):420.

Iyengar VK, Eisner T: Female choice increases offspring fitness in an arctiid moth (*Utetheisa ornatrix*). *Proceedings of the National Academy of Sciences* 1999a, 96(26), 15013-15016.

Iyengar VK, Eisner T: Heritability of body mass, a sexually selected trait, in an arctiid moth (*Utetheisa ornatrix*). *Proceedings of the National Academy of Sciences* 1999b, 96(16), 9169-9171.

Iyengar VK, Rossini C, Eisner T: Precopulatory assessment of male quality in an arctiid moth (*Utetheisa ornatrix*): Hydroxydanaidal is the only criterion of choice. *Behavioral Ecology and Sociobiology* 2001, 49(4), 283-288.

Jaklič D, Lapanje A, Zupančič K, Smrke D, Gunde-Cimerman N: Selective antimicrobial activity of maggots against pathogenic bacteria. *Journal of Medical Microbiology* 2008, 57(5):617-625.

Johansson BG, Jones TM: The role of chemical communication in mate choice. *Biological Reviews* 2007, 82(2):265-289.

Jones T, Quinnell R, Balmford A: Fisherian flies: benefits of female choice in a lekking sandfly. *Proceedings of the Royal Society of London B: Biological Sciences* 1998, 265(1406):1651-1657.

Jones T, Balmford A, Quinnell R: Adaptive female choice for middle-aged mates in a lekking sandfly. *Proceedings of the Royal Society of London B: Biological Sciences* 2000, 267(1444):681-686.

Juárez M, Ruiz M, Fernández P, Goane L, Villagrán M, Arce O, Armiñana A, Páez Jerez P, de la Vega M, Vera M, Groot AT: Communication interference in sympatrically occurring moth species. *Entomologia Experimentalis et Applicata* 2016, 158(1):25-33.

Kaltenpoth M, Strohm E: The scent of senescence: Age-dependent changes in the composition of the cephalic gland secretion of the male European beewolf, *Philanthus triangulum*. *Journal of Insect Science* 2006, 6.

Kaltenpoth M, Gottler W, Herzner G, Strohm E: Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 2005, 15:475-479.

Kaltenpoth M, Goettler W, Dale C, Stubblefield JW, Herzner G, Roeser-Mueller K, Strohm E: 'Candidatus Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. *International Journal of Systematic and Evolutionary Microbiology* 2006, 56:1403-1411.

Kaltenpoth M, Kroiss J, Strohm E: The odor of origin: kinship and geographical distance are reflected in the marking pheromone of male beewolves (*Philanthus triangulum* F., Hymenoptera, Crabronidae). *BMC Ecology* 2007, 7(1).

Kaltenpoth M, Schmitt T, Polidori C, Koedam D, Strohm E: Symbiotic streptomycetes in antennal glands of the South American digger wasp genus *Trachypus* (Hymenoptera, Crabronidae). *Physiological Entomology* 2010, 35(2):196-200.

Kaltenpoth M, Yildirim E, Guerbuez MF, Herzner G, Strohm E: Refining the Roots of the Beewolf-*Streptomyces* Symbiosis: Antennal Symbionts in the Rare Genus *Philanthinus* (Hymenoptera, Crabronidae). *Applied and Environmental Microbiology* 2012, 78(3):822-827.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Science of the United States* 2014, 111(17):6359-6364.

Karlson P, Butenandt A: Pheromones (ectohormones) in insects. *Annual Review of Entomology* 1959, 4(1):39-58.

Karlson P, Lüscher M: 'Pheromones': A new term for a class of biologically active substances. *Nature* 1959, 183(4653):55-56.

Klappert K, Reinhold K: Acoustic preference functions and sexual selection on the male calling song in the grasshopper *Chorthippus biguttulus*. *Animal Behaviour* 2003 65(1): 225-233.

Knight AL, Barros-Parada W, Bosch D, Escudero-Colomar L, Fuentes-Contreras E, Hernández-Sánchez J, Yung C, Kim Y, Kovanci O, Levi A: Similar worldwide patterns in the sex pheromone signal and response in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae). *Bulletin of Entomological Research* 2015, 105(01):23-31.

Koedam D, Slaa E, Biesmeijer J, Nogueira-Neto P: Unsuccessful attacks dominate a drone-preying wasp's hunting performance near stingless bee nests. *Genetics and Molecular Research* 2009, 8(2):690-702.

Koedam D, Morgan ED, Nunes TM, Patricio EFL, Imperatriz-Fonseca VL: Selective preying of the sphecid wasp *Trachypus boharti* on the meliponine bee *Scaptotrigona postica*: potential involvement of caste-specific cuticular hydrocarbons. *Physiological Entomology* 2011, 36(2):187-193.

Koehler S, Doubsky J, Kaltenpoth M: Dynamics of symbiont-mediated antibiotic production reveal efficient long-term protection for beewolf offspring. *Frontiers in Zoology* 2013, 10.

Kokko H, Brooks R, McNamara JM, Houston AI: The sexual selection continuum. *Proceedings of the Royal Society B: Biological Sciences* 2002, 269(1498):1331-1340.

Kokko H, Brooks R, Jennions MD, Morley J: The evolution of mate choice and mating biases. *Proceedings of the Royal Society B: Biological Sciences* 2003, 270(1515):653-664.

Kokko H, Jennions MD, Brooks R: Unifying and testing models of sexual selection. *Annual Review of Ecology, Evolution, and Systematics* 2006, 37:43-66.

Kolm N, Amcoff M, Mann RP, Arnqvist G: Diversification of a food-mimicking male ornament via sensory drive. *Current Biology* 2012, 22(15):1440-1443.

Kraaijeveld K, Kraaijeveld-Smit FJL, Maan ME: Sexual selection and speciation: the comparative evidence revisited. *Biological Reviews* 2011, 86(2):367-377.

Kroiss J, Schmitt T, Schreier P, Strohm E, Herzner G: A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 2006, 32(12):2763-2776.

Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, Strohm E, Svatos A: Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology* 2010a, 6(4):261-263.

Kroiss J, Lechner K, Strohm E: Male territoriality and mating system in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae): evidence for a "hotspot" lek polygyny. *Journal of Ethology* 2010b, 28(2):295-304.

Krombein KV: Biosystematic studies of Ceylonese wasps, VIII: A monograph of the Philanthidae (Hymenoptera: Sphecoidea). *Smithsonian Institution Press*, 1981.

Lande R: Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Science* 1981, 78(6):3721-3725.

Laurent P, Braekman J, Daloze D: Insect chemical defense. *Topics in Current Chemistry* 2005, 240:167-229.

Leary GP, Allen JE, Bunger PL, Luginbill JB, Linn CE, Macallister IE, Kavanaugh MP, Wanner KW: Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proceedings of the National Academy of Science* 2012, 109(35):14081-14086.

Lenoir A, Fresneau D, Errard C, Hefetz A: Individuality and colonial identity in ants: the emergence of the social representation concept. In: Information processing in social insects. Springer, 1999: 219-237.

Lewis SM, Cratsley CK, Demary K: Mate recognition and choice in *Photinus* fireflies. *Annales Zoologici Fennici* 2004, 41:809-821.

Löfstedt C: Population variation and genetic control of pheromone communication systems in moths. *Entomologia Experimentalis et Applicata* 1990, 54(3):199-218.

López P, Martín J: Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. *Biology Letters* 2005, 1(4):404-406.

Maan ME, Seehausen O: Ecology, sexual selection and speciation. *Ecology Letters* 2011, 14(6):591-602.

Marcillac, F, Grosjean, Y, Ferveur JF: A single mutation alters production and discrimination of *Drosophila* sex pheromones. *Proceedings of the Royal Society of London B: Biological Sciences* 2005, 272(1560):303-309.

Marco A, Chivers DP, Kiesecker JM, Blaustein AR: Mate choice by chemical cues in western redback (*Plethodon vehiculum*) and Dunn's (*P. dunni*) salamanders. *Ethology* 1998, 104(9):781-788.

Martín J, López P: Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. *Functional Ecology* 2006, 20(6):1087-1096.

McDaniel CA, Howard RW, Oneill KM, Schmidt JO: Chemistry of male mandibular gland secretions of *Philanthus basilaris* Cresson and *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1987, 13(2):227-235.

McDaniel CA, Schmidt JO, Howard RW: Mandibular gland secretions of the male beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1992, 18(1):27-37.

Milinski M, Griffiths S, Wegner KM, Reusch TB, Haas-Assenbaum A, Boehm T: Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. *Proceedings of the National Academy of Sciences of the United States of America* 2005, 102(12):4414-4418.

Moore AJ: Genetic evidence for the "good genes" process of sexual selection. *Behavioral Ecology and Sociobiology* 1994, 35(4):235-241.

Morgan ED: Chemical sorcery for sociality: exocrine secretions of ants (Hymenoptera: Formicidae). *Myrmecological News* 2008, 11:79-90.

Morrow EH, Pitcher TE, Arnqvist G: No evidence that sexual selection is an 'engine of speciation' in birds. *Ecology Letters* 2003, 6(3):228-234.

Niehuis O, Buellesbach J, Gibson JD, Pothmann D, Hanner C, Mutti NS, Judson AK, Gadau J, Ruther J, Schmitt T: Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* 2013, 494(7437):345-348.

Noirot C, Quennedey A: Fine-structure of insect epidermal glands. *Annual Review of Entomology* 1974, 19:61-80.

Noirot C, Quennedey A: Glands, gland cells, glandular units: Some comments on terminology and classification. *Annales de la Société entomologique de France* 1991, 123-128.

O`Neill KM: Solitary wasps: behavior and natural history. 1st ed. Ithaca: Cornell University Press, 2001.

Ord TJ, Martins EP: Tracing the origins of signal diversity in anole lizards: phylogenetic approaches to inferring the evolution of complex behaviour. *Animal Behaviour* 2006, 71(6):1411-1429.

Otti O, Tragust S, Feldhaar H: Unifying external and internal immune defences. *Trends in Ecology & Evolution* 2014, 29(11):625-634.

Osada K, Yamazaki K, Curran M, Bard J, Smith BP, Beauchamp GK: The scent of age. *Proceedings of the Royal Society of London B: Biological Sciences* 2003, 270(1518):929-933.

Panhuis TM, Butlin R, Zuk M, Tregenza T: Sexual selection and speciation. *Trends in Ecology & Evolution* 2001, 16(7):364-371.

Pedrini N, Ortiz-Urquiza A, Huarte-Bonnet C, Fan Y, Juarez P, Keyhani NO: Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. *Proceedings of the National Academy of Sciences of the United States of America* 2015, 112(28):E3651-E3660.

Penagos-Arévalo AC, Billen J, Sarmiento CE: Uncovering head gland diversity in neotropical Polistinae wasps (Hymenoptera, Vespidae): Comparative analysis and description of new glands. *Arthropod Structure & Developemt* 2015, 44(5):415-425.

Pietrobon TAO, Caetano FH: Ultramorphology and histology of the ectal mandibular gland in *Polistes versicolor* (Olivier) (Hymenoptera: Vespidae). *Cytologia* 2003, 68(1):89-94.

Polidori C, Boesi R, Isola F, Andrietti F: Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): the role of prey size. *European Journal of Entomology* 2005, 102(4):801-804.

Polidori C, Federici M, Papadia C, Andrietti F: Nest sharing and provisioning activity of females of the digger wasp, *Cerceiis rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 2006, 73(1):55-65.

Polidori C, Boesi R, Pesarini C, Papadia C, Bevacqua S, Federici M, Andrietti F: Temporal relationship between the prey spectrum and population structure of the weevil-hunting wasp *Cerceris arenaria* (Hymenoptera: Crabronidae). *Zoological Studies* 2007, 46(1):83-91.

Polidori C, Gobbi M, Chatenoud L, Santoro D, Montani O, Andrietti F: Taxon-biased diet preference in the 'generalist' beetle-hunting wasp *Cerceris rubida* provides insights on the evolution of prey specialization in apoid wasps. *Biological Journal of the Linnean Society* 2010, 99(3):544-558.

Pomiankowski A, Iwasa Y: Runaway ornament diversity caused by Fisherian sexual selection. *Proceedings of the National Academy of Sciences of the United States of America* 1998, 95(9):5106-5111.

Pöppel A-K, Koch A, Kogel K-H, Vogel H, Kollewe C, Wiesner J, Vilcinskas A: Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly *Lucilia sericata*. *Biological Chemistry* 2014, 395(6):649-656.

Prendeville HR, Stevens L: Microbe inhibition by *Tribolium* flour beetles varies with beetle species, strain, sex, and microbe group. *Journal of Chemical Ecology* 2002, 28(6):1183-1190.

Price JJ, Friedman NR, Omland KE: Song and plumage evolution in the new world orioles (*Icterus*) show similar lability and convergence in patterns. *Evolution* 2007, 61(4):850-863.

Proulx SR: Female choice via indicator traits easily evolves in the face of recombination and migration. *Evolution* 2001, 55(12):2401-2411.

Prum RO: Phylogenetic tests of alternative intersexual selection mechanisms: Trait macroevolution in a polygynous clade (Aves: Pipridae). *The American Naturalist* 1997, 149(4):668-692.

Pulawski WJ: Number of species. In: Catalog of Sphecidae. California Academy of Sciences, San Francisco. 2017. http://researcharchive.calacademy.org/research/entomology/entomology_resources/hymenoptera/sphecidae/number_of_species.pdf. Accessed 28 March 2017.

Quennedey A: Insect epidermal gland cells: Ultrastructure and morphogenesis. *Microscopic Anatomy of Invertebrates* 1998, 11:177-207.

Quicke D: Parasitic wasps. London: Chapman and Hall, 1997.

Rantala MJ, Jokinen I, Kortet R, Vainikka A, Suhonen J: Do pheromones reveal male immunocompetence? *Proceedings of the Royal Society of London B: Biological Sciences* 2002, 269(1501):1681-1685.

Rantala MJ, Kortet R, Kotiaho JS, Vainikka A, Suhonen J: Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Functional Ecology* 2003, 17(4):534-540.

Rathmayer W: Das Paralysierungsproblem beim Bienenwolf *Philanthus triangulum* F. (Hym. Sphec.). *Zeitschrift für vergleichende Physiologie* 1962, 6:105-122.

Reinhold K: Modeling a version of the good-genes hypothesis: female choice of locally adapted males. *Organisms Diversity & Evolution* 2004, 4(3):157-163.

Reinhold K: Variation in acoustic signalling traits exhibits footprints of sexual selection. *Evolution* 2011, 65(3):738-745.

Ristich SS: The host relationship of a miltogrammid fly *Senotainia trilineata* (VDW). *Ohio Journal of Science* 1956, 56(5):271-274.

Ritchie MG: Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics* 2007, 38:79-102.

Roelofs WL, Glover T, Tang X-H, Sreng I, Robbins P, Eckenrode C, Löfstedt C, Hansson BS, Bengtsson BO: Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proceedings of the National Academy of Science* 1987, 84(21):7585-7589.

Roelofs WL, Liu W, Hao G, Jiao H, Rooney AP, Linn CE: Evolution of moth sex pheromones via ancestral genes. *Proceedings of the National Academy of Science* 2002, 99(21):13621-13626.

Roeser-Mueller K, Strohm E, Kaltenpoth M: Larval rearing temperature influences amount and composition of the marking pheromone of the male beewolf, *Philanthus triangulum*. *Journal of Insect Science* 2010, 10(1):74.

Rozen DE, Engelmoer DJP, Smiseth PT: Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences of the United States of America* 2008, 105(46):17890-17895.

Ryan MJ: Sexual selection, sensory systems and sensory exploitation. *Oxford Surveys in Evolutionary Biology* 1990, 7:157-195.

Ryan MJ: Sexual selection, receiver biases, and the evolution of sex differences. *Science* 1998, 281(5385):1999-2003.

Ryan MJ, Cummings ME: Perceptual biases and mate choice. *Annual Review of Ecology, Evolution, and Systematics* 2013, 44:437-459.

Schmidt JO, Oneill KM, Fales HM, McDaniel CA, Howard RW: Volatiles from mandibular glands of male beewolves (Hymenoptera, Sphecidae, *Philanthus*) and their possible roles. *Journal of Chemical Ecology* 1985, 11(7):895-901.

Schmidt JO, McDaniel CA, Thomas RTS: Chemistry of Male Mandibular Gland Secretions of *Philanthus triangulum*. *Journal of Chemical Ecology* 1990, 16(7):2135-2143.

Schmitt T, Strohm E, Herzner G, Bicchi C, Krammer G, Heckel F, Schreier P: (*S*)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum. Journal of Chemical Ecology* 2003, 29(11):2469-2479.

Schmitt T, Herzner G, Weckerle B, Schreier P, Strohm E: Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. *Apidologie* 2007, 38(2):164-170.

Scholes E: Evolution of the courtship phenotype in the bird of paradise genus *Parotia* (Aves: Paradisaeidae): homology, phylogeny, and modularity. *Biological Journal of the Linnean Society* 2008, 94(3):491-504.

Seddon N, Merrill RM, Tobias JA: Sexually selected traits predict patterns of species richness in a diverse clade of suboscine birds. *The American Naturalist* 2008, 171(5):620-631.

Seddon N, Botero CA, Tobias JA, Dunn PO, MacGregor HEA, Rubenstein DR, Uy JAC, Weir JT, Whittingham LA, Safran RJ: Sexual selection accelerates signal evolution during speciation in birds. *Proceedings of the Royal Society B: Biological Sciences* 2013, 280(1766).

Seehausen O, van Alphen JM: Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecology Letters* 1999, 2(4):262-271.

Shohet AJ, Watt PJ: Female association preferences based on olfactory cues in the guppy, *Poecilia reticulata*. *Behavioral Ecology and Sociobiology* 2004, 55(4):363-369.

Simmons LW: Sexual selection and genital evolution. *Austral Entomology* 2014, 53(1):1-17.

Simon-Thomas R, Poorter E: Notes on the behaviour of males of *Philanthus triangulum* (F.) (Hymenoptera, Sphecidae). *Tijdschrift voor Entomologie* 1972, 115:141-151.

Simon-Thomas RT, Veenendaal RL: Observations on the underground behavior of *Philanthus triangulum* (Hymenoptera, Sphecidae). *Entomologische Berichten Amsterdam* 1978, 38 (1):3-8.

Slessor KN, Kaminski LA, King GGS, Borden JH, Winston ML: Semiochemical Basis of the Retinue Response to Queen Honey Bees. *Nature* 1988, 332(6162):354-356.

Slessor KN, Winston ML, Le Conte Y: Pheromone communication in the honeybee (*Apis mellifera* L.). *Journal of Chemical Ecology* 2005, 31(11):2731-2745.

Smadja C, Butlin RK: On the scent of speciation: The chemosensory system and its role in premating isolation. *Heredity* 2009, 102(1):77-97.

Snook RR: The evolution of polyandry. In: Shuker D, Simmons L, eds.: The evolution of insect mating systems. Oxford University Press, Oxford: Oxford University Press, 2014: 159-180.

Steiger S, Stökl J: The role of sexual selection in the evolution of chemical signals in insects. *Insects* 2014, 5(2):423-438.

Steiger S, Schmitt T, Schaefer HM: The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society B: Biological Sciences* 2010, 278(1708):970-979.

Steiner AL: Observations on spacing, aggressive and lekking behavior of digger wasp males of *Eucerceris flavocincta* (Hymenoptera: Sphecidae, Cercerini). *Journal of the Kansas Entomological Society* 1978, 51(3):492-498.

Stökl J, Herzner G: Morphology and ultrastructure of the allomone and sex-pheromone producing mandibular gland of the parasitoid wasp *Leptopilina heterotoma* (Hymenoptera: Figitidae). *Arthropod Structure & Development* 2016, 45(4):333-340.

Strohm E: Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). Berlin: Verlag Dr, Köster, 1995.

Strohm E: Factors affecting body size and fat content in a digger wasp. *Oecologia* 2000, 123(2):184-191.

Strohm E, Lechner K: Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 2000, 59(1):183-191.

Strohm E, Linsenmair KE: Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence*, Zoology Jena* 1994/1995, 98:137-146.

Strohm E, Linsenmair KE: Low resource availability causes extremely male-biased investment ratios in the European beewolf, *Philanthus triangulum* F (Hymenoptera, Sphecidae). *Proceedings of the Royal Society B: Biological Sciences* 1997, 264:423-9.

Strohm, E, Linsenmair, KE: Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 1999, 47:76-88.

Strohm E, Linsenmair KE: Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biological Journal of the Linnean Society* 2000, 69 (2):173-192.

Strohm E, Linsenmair KE: Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 2001, 26(2):198-203.

Strohm E, Marliani A: The cost of parental care: Prey hunting in a digger wasp. *Behavioral Ecology* 2002, 13:52-8.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36(2):113-122.

Strohm E, Herzner G, Kaltenpoth M, Boland W, Schreier P, Geiselhardt S, Peschke K, Schmitt T: The chemistry of the postpharyngeal gland of female European beewolves. *Journal of Chemical Ecology* 2008, 34(5):575-583.

Symonds MR, Elgar MA: The mode of pheromone evolution: evidence from bark beetles. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 2004, 271(1541):839-846.

Symonds MR, Elgar MA: The evolution of pheromone diversity. *Trends in Ecology & Evolution* 2008, 23(4):220-228.

Symonds MR, Moussalli A, Elgar MA: The evolution of sex pheromones in an ecologically diverse genus of flies. *Biological Journal of the Linnean Society* 2009, 97(3):594-603.

Tabata, J, Ishikawa Y: Genetic basis to divergence of sex pheromones in two closely related moths, *Ostrinia scapulalis* and *O. zealis*. *Journal of Chemical Ecology* 2005, 31(5):1111-1124.

Thornhill R: Female preference for the pheromone of males with low fluctuating asymmetry in the Japanese scorpionfly (*Panorpa japonica*: Mecoptera). *Behavioral Ecology* 1992, 3(3):277-283.

Thornhill R, Gangestad SW: The scent of symmetry: A human sex pheromone that signals fitness? *Evolution and Human Behavior* 1999, 20(3):175-201.

Tinbergen N: Über die Orientierung des Bienenwolfes (*Philanthus triangulum* Fabr.). *Zeitschrift für vergleichende Physiologie* 1932, 16:305 - 334.

Tinbergen N: Über die Orientierung des Bienenwolfes. II. Die Bienenjagd. *Zeitschrift für vergleichende Physiologie* 1935, 21:688-716.

Tragust S: External immune defence in ant societies (Hymenoptera: Formicidae): The role of antimicrobial venom and metapleural gland secretion. *Myrmecological News* 2016, 23:119-128.

Trivers RL: Parental investment and sexual selection. In: Campbell B ed.: Sexual selection and the descent of man: The Darwinian pivot. Chicago: Aldine, 1972: 136-179.

van Doorn GS, Dieckmann U, Weissing FJ: Sympatric speciation by sexual selection: a critical reevaluation. *The American Naturalist* 2004, 163(5):709-725.

Vinson SB, Williams HJ, Frankie GW, Wheeler JW, Blum MS, Coville RE: Mandibular glands of male *Centris adani* (Hymenoptera, Anthophoridae) - Their morphology, chemical constituents, and function in scent marking and territorial behavior. *Journal of Chemical Ecology* 1982 ,8(2):319-327.

Vinson SB, Williams HJ, Frankie GW, Coville RE: Comparative morphology and chemical contents of male mandibular glands of several *Centris* species (Hymenoptera, Anthophoridae) in Costa Rica. *Comparative Biochemistry and Physiology A* 1984, 77(4):685-688.

Wagner CE, Harmon LJ, Seehausen O: Ecological opportunity and sexual selection together predict adaptive radiation. *Nature* 2012, 487(7407):366-370.

Wcislo WT, Fernández-Marín H, Di Trani JC: Use of communal nests by male and female *Trachypus petiolatus* (Hymenoptera: Sphecidae). *Journal of the Kansas Entomological Society* 2004, 77(4):323-331.

Weber MG, Mitko L, Eltz T, Ramírez SR: Macroevolution of perfume signalling in orchid bees. *Ecology Letters* 2016, 19(11):1314-1323.

Weiss K, Parzefall C, Herzner G: Multifaceted defense against antagonistic microbes in developing offspring of the parasitoid wasp *Ampulex compressa* (Hymenoptera, Ampulicidae). *PLoS ONE* 2014, 9(6).

Wenzel JW: Male reproductive behavior and mandibular glands in *Polistes major* (Hymenoptera: Vespidae). *Insectes Sociaux* 1987, 34(1):44-57.

Whittaker DJ, Gerlach NM, Soini HA, Novotny MV, Ketterson ED: Bird odour predicts reproductive success. *Animal Behaviour* 2013, 86(4):697-703.

Wheeler WM: A solitary wasp (*Aphilanthops Frigidus* F. Smith) that provisions its nest with queen ants. J *Animal Behaviour* 1913, 3(5):374.

Wicker-Thomas C: Pheromonal communication involved in courtship behavior in Diptera. *Journal of Insect Physiology* 2007, 53(11):1089-1100.

Wiley C, Ellison CK, Shaw KL: Widespread genetic linkage of mating signals and preferences in the Hawaiian cricket *Laupala*. *Proceedings of the Royal Society of London B: Biological Sciences* 2011.

Willers D, Lehmann-Danzinger H, Führer E: Antibacterial and antimycotic effect of a newly discovered secretion from larvae of an endoparasitic insect, *Pimpla turionellae*. *Archives of Microbiology* 1982, 133:225-229.

Willis C, Poulin R: Preference of female rats for the odours of non-parasitised males: The smell of good genes? *Folia Parasitologica* 2000, 47(1):6-10.

Wilson EO, Regnier Jr FE: The evolution of the alarm-defense system in the formicine ants. *The American Naturalist* 1971, 105(943):279-289.

Winston ML, Slessor KN: The Essence of Royalty - Honey-Bee Queen Pheromone. *American Scientist* 1992, 80(4):374-385.

Winston M, Slessor K, Willis L, Naumann K, Higo H, Wyborn M, Kaminski L: The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.). *Insectes Sociaux* 1989, 36(1):15-27.

Wyatt TD: Pheromones and animal behaviour: Communication by smell and taste: Cambridge University Press, 2003.

Wyatt TD: Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A* 2010, 196(10):685-700.

Yasui Y: The 'genetic benefits' of female multiple mating reconsidered. *Trends in Ecology & Evolution* 1998, 13(6):246-250.

Zahavi A: Mate selection - a selection for a handicap. *Journal of theoretical Biology* 1975, 53(1):205-214.

Zahavi A: The cost of honesty: further remarks on the handicap principle. *Journal of theoretical Biology* 1977, 67(3):603-605.

Zimmermann D, Vilhelmsen L: The sister group of Aculeata (Hymenoptera) - Evidence from internal head anatomy, with emphasis on the tentorium. *Arthropod Systematics & Phylogeny* 2016, 74(2):195-218.

# CHAPTER 2

## COMPARATIVE MORPHOLOGY OF THE POSTPHARYNGEAL GLAND IN THE PHILANTHINAE (HYMENOPTERA, CRABRONIDAE) AND THE EVOLUTION OF AN ANTIMICROBIAL BROOD PROTECTION MECHANISM

*BMC Evolutionary Biology (2015), doi: 10.1186/s12862-015-0565-0*

Katharina Weiss, Erhard Strohm, Martin Kaltenpoth, Gudrun Herzner

### 2.1 Summary

**Background:**
Hymenoptera that mass-provision their offspring have evolved elaborate antimicrobial strategies to ward off fungal infestation of the highly nutritive larval food. Females of the Afro-European *Philanthus triangulum* and the South American *Trachypus elongatus* (Crabronidae, Philanthinae) embalm their prey, paralyzed bees, with a secretion from a complex postpharyngeal gland (PPG). This coating consists of mainly unsaturated hydrocarbons and reduces water accumulation on the prey's surface, thus rendering it unfavorable for fungal growth. Here we (1) investigated whether a North American *Philanthus* species also employs prey embalming and (2) assessed the occurrence and morphology of a PPG among females of the subfamily Philanthinae in order to elucidate the evolution of prey embalming as an antimicrobial strategy.

**Results:**
We provide clear evidence that females of the North American *Philanthus gibbosus* possess large PPGs and embalm their prey. The comparative analyses of 26 species from six genera of the Philanthinae, using histological methods and 3D-reconstructions, revealed pronounced differences in gland morphology within the subfamily. A formal statistical analysis based on defined characters of the glands confirmed that while all members of the derived tribe Philanthini have large and complex PPGs, species of the two more basal tribes, Cercerini and Aphilanthopsini, possess simple and comparatively small glands. According to an ancestral state reconstruction, the complex PPG most likely evolved in the last common ancestor of the Philanthini, thus representing an autapomorphy of this tribe.

**Conclusion:**
Prey embalming, as described for *P. triangulum* and *T. elongatus*, and now also for *P. gibbosus*, most probably requires a complex PPG. Hence, the morphology and size of the PPG may allow for inferences about the origin and distribution of the prey embalming behavior within the Philanthinae. Based on our results, we suggest that prey embalming has evolved as an antimicrobial strategy in and is restricted to the tribe Philanthini, which seems to face exceptional threats with regard to fungal infestations of their larval provisions.

## 2.2 Introduction

Microorganisms pose serious threats to insects both as pathogens (Hajek & St Leger 1994, Sikorowski et al. 2001, Secil et al. 2012) and food competitors (Janzen 1977, Burkepile et al. 2006, Trienens et al. 2010). Many solitary wasps rely on paralyzed arthropod prey as food for their developing offspring (O`Neill 2001). To protect these nutrient rich resources from harmful microorganisms, wasps have evolved elaborate antimicrobial strategies (e.g. Willers et al. 1982, Führer & Willers 1986, Strohm & Linsenmair 2001, Kaltenpoth et al. 2005, Herzner et al. 2013a, Kaltenpoth & Engl 2014, Weiss et al. 2014). Especially in species that mass-provision their brood with paralyzed prey, it is crucial that the stored resources stay consumable throughout the feeding period of the larva (Vander Wall 1990).

The European beewolf *Philanthus triangulum* (Fabricius) (Hymenoptera, Crabronidae, Philanthinae) shows a remarkable antimicrobial defense mechanism. Females of this digger wasp prey exclusively on honeybee workers, *Apis mellifera*, as provisions for their offspring (see e.g. Strohm & Linsenmair 1997, Strohm & Marliani 2002). The paralyzed bees are stored in subterranean brood cells under warm and humid conditions, which impose a high risk of fungal infestation on the larval provisions (e.g. Strohm & Linsenmair 2001). As a countermeasure, females extensively lick the surface of their prey prior to oviposition (Strohm & Linsenmair 2001), thereby applying large amounts of a lipid secretion to the bee's cuticle (Herzner & Strohm 2007, Herzner et al. 2007), a behavior termed 'embalming' in the following. Since this secretion contains predominantly unsaturated hydrocarbons (HCs) (Herzner et al. 2007, Strohm et al. 2008), embalming not only increases the total amount of HCs but also the proportion of unsaturated HCs on the prey's surface (Herzner & Strohm 2007, Herzner et al. 2007). The coating of unsaturated HCs changes the physicochemical properties of the bees' epicuticle, resulting in a reduction of water condensation. The resulting change in microclimate retards fungal growth, thus reducing the decomposition of the larval resources and increasing larval survival (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2011a).

The source of the embalming secretion is the postpharyngeal gland (PPG) (Herzner et al. 2007, Strohm et al. 2007, 2008), a cephalic gland which has long been thought to be restricted to ants where it is mainly involved in the generation and maintenance of the colony odor (e.g. Soroker et al. 1994, 1995, Hefetz et al. 1996, Lenoir et al. 1999; for a review of other functions see Eelen et al. 2006). In female *P. triangulum*, the PPG consists of two large reservoirs originating dorsally from the pharynx at the proximal end of the hypopharyngeal plate and extending laterally

anterior to the brain (hereafter referred to as 'upper part of the PPG') (Strohm et al. 2007). Each reservoir of this upper part of the gland consists of a main root with numerous 'fingers' branching off, resulting in an overall glove-like shape of the gland (Strohm et al. 2007). Additionally, a smaller unpaired sac-like evagination extends ventrally from the pharynx (hereafter referred to as 'lower part of the PPG') (Strohm et al. 2007). The walls of all parts of the PPG are formed by a monolayered epithelium with apical hairs that reach into the lumen of the gland. The content of the gland is most probably not synthesized by the epithelial cells but is rather sequestered from the hemolymph via the enlarged gland surface (Strohm et al. 2007, Strohm et al. 2010).

Besides *P. triangulum*, a PPG has also been described for two species of the genus *Trachypus* (Herzner et al. 2013b) (which group within *Philanthus* according to a recent phylogenetic analysis, Kaltenpoth et al. 2014). The morphology of the PPGs of both *Trachypus elongatus* and *Trachypus boharti* closely resembles the PPG of *P. triangulum* (Herzner et al. 2013b). In both *Trachypus* species, the PPG also contains mostly HCs, and *T. elongatus* has been shown to embalm its prey, stingless bees, with the secretion of its PPG (Herzner et al. 2013b). Both species for which prey embalming has so far been described, *P. triangulum* and *T. elongatus*, belong to the tribe Philanthini within the subfamily Philanthinae (Alexander 1992, Kaltenpoth et al. 2014). The Philanthinae consist of eight genera separated into three tribes (Alexander 1992): The Philanthini, comprising (*Philanthus* + *Trachypus*) + *Philanthinus*, represent a sister group to the other two tribes, the Cercerini, comprising (*Cerceris* + *Eucerceris*) + *Pseudoscolia*, and the Aphilanthopsini comprising *Clypeadon* + *Aphilanthops* (Alexander 1992). All philanthine wasps share basic life-history traits, including hunting and nesting behavior. Females build subterranean nests and mass-provision brood cells with paralyzed insects (Hymenoptera or Coleoptera) as food for the developing larvae (e.g. Evans 1962, Bohart & Menke 1976, Evans & O'Neill 1988, Polidori et al. 2005, 2006). Thus, all Philanthinae may face similar selection pressures with regard to the protection of their larval provisions and their offspring against detrimental microbes. As a consequence, all of these species likely either employ prey embalming with PPG secretion or some other prey preservation mechanism.

In the present study, we aim to shed light on the evolution of the prey embalming behavior and the associated complex PPG and ask whether these traits are common to all Philanthinae or have arisen in only some lineages. First, in order to broaden our knowledge about the distribution of this antimicrobial mechanism within the Philanthinae, we analyzed whether the North American *Philanthus gibbosus* (Fabricius), shows prey embalming. Second, we investigated 26 species belonging to six genera representing all three tribes of the Philanthinae with regard to the

occurrence and morphology of the PPG as well as another head gland that could be involved in prey preservation, the mandibular gland (MG). We provide a comparative morphological analysis based on characters obtained by histological investigations and 3D-reconstructions of the head glands. Our analysis revealed pronounced differences in the morphology of the PPG between the different tribes of the Philanthinae, which may allow for inferences about the origin and distribution of the prey embalming behavior within this subfamily.

## 2.3 Methods

### 2.3.1 Prey embalming in *Philanthus gibbosus*

A detailed description of the methodology of this section is given in the Supplementary Material (section 2.6.1). Briefly, *P. gibbosus* females were reared in observation cages as described earlier for *P. triangulum* (Strohm & Linsenmair 1994) and supplied with halictid bees (Hymenoptera, Halictidae) as prey. Owing to the limited availability of halictid bees, several different species had to be used. To investigate whether *P. gibbosus* females embalm their prey with HCs from their PPG, paralyzed bees were removed from *P. gibbosus* brood cells (hereafter referred to as 'provisioned bees', N = 6) and their cuticular HCs were analyzed by gas chromatography/mass spectrometry (GC/MS). For comparison, halictid bees which had no contact to the beewolves (hereafter referred to as 'control bees', N = 9) and heads of field-caught *P. gibbosus* females (N = 4) were analyzed accordingly. As both provisioned and control bees comprised a number of different halictid species, a molecular identification of the species was conducted (see Table S2.1, Supplementary Material section 2.6.1). For the chemical analysis, whole bees as well as *P. gibbosus* heads were extracted, an internal standard was added for quantification and aliquots were analyzed by GC/MS. N-alkanes were identified by comparison of their retention times and mass spectra to synthetic references. Linear retention indices (LRIs) for all other substances were calculated in relation to the n-alkanes (Van den Dool & Kratz 1963), and alkenes were identified by their LRIs and mass spectra as described in Strohm et al. (2008). The structure of the unsaturated ketone nonacosen-6-one was tentatively assigned by its mass spectrum as described previously (Herzner et al. 2013b). Absolute amounts of components were calculated by use of the internal standard and compared between provisioned and control bees by a Mann–Whitney U test. Relative amounts were calculated by standardizing the total peak area of a sample to 100 %. The proportions of unsaturated compounds were *arcsine*-transformed and compared between provisioned and control bees using a *t* test; in addition, we compared the relative amounts of

individual HCs. All tests were performed using the statistics software package PAST (Version 2.08b) (Hammer et al. 2001). Unless otherwise stated, values given are means ± standard deviation (SD).

### 2.3.2 Comparative morphology of head glands

#### *2.3.2.1 Specimens*

Females of 26 species and subspecies belonging to six genera covering the three tribes of the crabronid subfamily Philanthinae were included in the morphological analysis (Table 2.1). The tribe Philanthini was represented by eight *Philanthus* species from Europe and South Africa, nine *Philanthus* species from North America, four *Trachypus* species from South America, and one *Philanthinus* species from Turkey. The Aphilanthopsini were represented by one *Clypeadon* and one *Aphilanthops* species from North America, and the Cercerini by two European *Cerceris* species.

#### *2.3.2.2 Histology*

Wasps were cold anesthetized and decapitated. The heads were fixed using different fixing agents (Table 2.1). For large heads, both compound eyes were laterally cut off after fixation using sharp razor blades to facilitate the infiltration of the embedding medium. Heads were then thoroughly rinsed in 70 % or (in case of formalin-ethanol-acetic acid fixation) 80 % ethanol, dehydrated in a graded ethanol series and propylene oxide, and embedded in Epon 812 according the suppliers instructions (Polysciences Europe GmbH, Eppelheim, Germany). Sagittal semithin sections (4 µm) were cut with a microtome (Reichert Ultracut; Leica Microsystems AG, Wetzlar, Germany) equipped with a diamond knife and subsequently stained with toluidine blue (Adam & Czihak 1964). The resulting series of histological sections were used for histological investigation of the head glands by light microscopy (Zeiss Axiophot 2; Carl Zeiss Microscopy GmbH, Jena, Germany) using bright field and differential interference contrast settings and for 3D-reconstructions of the head glands.

#### *2.3.2.3 3D-Reconstruction*

To visualize the overall morphology of head glands and facilitate their comparison, 3D-reconstructions of the head glands were conducted for 22 of the 26 investigated species. Even

though for one *Trachypus* and three *Philanthus* species no complete series of sections were available (Table 2.1), the histological sections were sufficient to allow for the determination of gland characters. For 3D-reconstruction, continuous series of semithin sections of one individual per species were photographed using a digital microscope camera (Olympus DP20; Olympus, Japan) attached to a light microscope (Zeiss Axiophot 2; Carl Zeiss Microscopy GmbH, Jena, Germany). The digitalized sections were automatically aligned with regard to each other using the software package TrakEM2 (Cardona et al. 2012) for the open source image processing software Fiji (Schindelin et al. 2012), and the alignment was subsequently corrected manually where necessary. The relevant structures within the head capsule were then marked as 3D-objects in TrakEM2 by manually outlining them in each picture of a series. Finally, 3D-reconstructions were calculated and visualized using Fiji's 3D-viewer plug-in (Schmid et al. 2010).

**Table 2.1** List of species included in the comparative morphological study of head glands of female Philanthinae, including information on the number of specimens examined (N), collection site of the species (Country), the fixative agent used for the histological preparation (Fixative; AAF = formalin-ethanol-acetic acid; Bouin = alcoholic Bouin; EtOH = 100 % ethanol), and whether a 3D-reconstruction of the head glands was conducted (3D; yes = 3D-reconstruction; no = glands not reconstructed).

| Species | N | Country | Fixative | 3D |
|---|---|---|---|---|
| ***Aphilanthops frigidus*** | 2 | USA | AAF | yes |
| ***Clypeadon laticinctus*** | 3 | USA | AAF | yes |
| ***Cerceris arenaria*** | 2 | Germany | Bouin | yes |
| ***Cerceris quinquefasciata*** | 3 | Germany | AAF | yes |
| ***Philanthinus quattuordecimpunctatus*** | 3 | Turkey | EtOH | yes |
| ***Trachypus flavidus*** | 1 | Brazil | EtOH | yes |
| ***Trachypus elongatus*** | 3 | Brazil | AAF | yes |
| ***Trachypus patagonensis*** | 1 | Brazil | AAF | no |
| ***Trachypus boharti*** | 3 | Brazil | AAF | yes |
| ***Philanthus venustus*** | 1 | Turkey | AAF | yes |
| ***Philanthus coronatus*** | 1 | Croatia | Bouin | yes |
| ***Philanthus triangulum triangulum*** | 3 | Germany | Bouin | no |
| ***Philanthus triangulum diadema*** | 3 | South Africa | AAF | yes |
| ***Philanthus capensis*** | 1 | South Africa | AAF | yes |
| ***Philanthus loefflingi*** | 3 | South Africa | AAF | yes |
| ***Philanthus rugosus*** | 3 | South Africa | AAF | yes |
| ***Philanthus melanderi*** | 1 | South Africa | AAF | yes |
| ***Philanthus bicinctus*** | 1 | USA | Bouin | yes |
| ***Philanthus ventilabris*** | 2 | USA | AAF | yes |
| ***Philanthus crabroniformis*** | 1 | USA | AAF | no |
| ***Philanthus multimaculatus*** | 1 | USA | AAF | yes |
| ***Philanthus barbiger*** | 2 | USA | AAF | yes |
| ***Philanthus gibbosus*** | 3 | USA | Bouin | yes |
| ***Philanthus albopilosus*** | 2 | USA | Bouin | yes |
| ***Philanthus psyche*** | 2 | USA | AAF | yes |
| ***Philanthus pulcher*** | 1 | USA | AAF | no |

*2.3.2.4 Statistical analysis of gland morphology*

After a comprehensive examination of both semithin histological sections and 3D-reconstructions of the head glands of female Philanthinae, we defined eight morphological characters of the PPG for a comparative statistical analysis. As the PPG might not be the only possible source of the embalming secretion (see Discussion), we also included five morphological characters of the MG, the only other head gland of female Philanthinae with a reservoir of considerable size. The 13 characters of the two glands comprised information on gland structure, as well as their overall shape and location within the head capsule. For each character, the different character states were categorized and numerically coded for statistical analysis. Detailed descriptions of all characters and the coding of their different states are given in the Supplementary Material (section 2.6.2). In short, the defined characters were: (1) overall structure of the PPG, (2) shape of the upper part of the PPG, (3) number of lobes of the upper part of the PPG, (4) number of openings of the upper part of the PPG to the pharynx, (5) relative size of the PPG in relation to the head capsule, (6) structure of the inner walls of the PPG, (7) shape of the lower part of the PPG, (8) number of openings of the lower part of the PPG to the pharynx, (9) overall structure of the MG, (10) relative size of the MG in relation to the head capsule, (11) branching of the MG, (12) structure of the inner walls of the MG, (13) association of the MG with gland cells.

To formally assess the pattern of PPG morphology among the species we used characters 1 through 8 of the data matrix (Table S2.2, Supplementary Material section 2.6.2) to conduct a hierarchical cluster analysis for ordination with the statistics software package PAST (Version 2.08b) (Hammer et al. 2001). In a second analysis, the morphological characters of the MG were included (Table S2.2, characters 9 through 13) in order to consider a possible impact of MG morphology on the clustering of species. Bray-Curtis indices were used as similarity measures and 'unweighted pair-group averages' as the clustering algorithm; the number of bootstrap replicates was set to 10,000.

To trace the evolution of PPG overall shape, the character which shows the most striking differences among the Philanthinae and most probably reflects differences in function of the PPG (see Results und Discussion), we conducted an ancestral state reconstruction (Pagel 1999) based on the molecular phylogeny of the Philanthinae (Kaltenpoth et al. 2014). The ancestral state reconstruction was performed using both maximum parsimony (unordered character states) and maximum likelihood (ML) approaches in Mesquite (Version 3.04) (Maddison & Maddison 2015). The ML analysis was performed using the Markov k-state 1 parameter model, in which any

particular character change is considered equally probable, as the model of evolution. As outgroup we included the only apoid wasp outside the Philanthinae currently known to possess a PPG, the cockroach wasp *Ampulex compressa* (Fabricius) (Hymenoptera, Ampulicidae) (Herzner et al. 2011b). In *A. compressa* the PPG is located behind the brain (as opposed to is location anterior to the brain in the Philanthinae), but it shows the typical structure (monolayered epithelium, hairs), ultrastructure of the epithelial cells, and chemistry (HCs) (Herzner et al. 2011b) that can be found in both, the Philanthinae (Strohm et al. 2007, 2008, Herzner et al. 2013b) and ants (Soroker et al. 1994, 1995, Hefetz et al. 1996, Lenoir et al. 1999, Eelen et al. 2006). With reference to a recent concept of homology (Wagner 2014), which proposes that the position of homologous characters can be variable among species, we tentatively assume homology between the PPGs of *A. compressa* and the Philanthinae.

## 2.4 Results

### 2.4.1 Prey embalming in *Philanthus gibbosus*

To establish whether *P. gibbosus* females embalm their prey, the chemical profiles of *P. gibbosus* females' heads, surfaces of provisioned bees taken from *P. gibbosus* brood cells, and those of control bees caught in the field were compared. In head extracts of *P. gibbosus* females, we detected 19 compounds, including 17 alkanes, alkenes, and alkadienes with chain lengths ranging from 23 to 31 carbon atoms, as well as the unsaturated long-chain ketone nonacosen-6-one, and one unidentified compound (Table S2.3, Supplementary Material section 2.6.3). The extracts were dominated by unsaturated HCs (mean proportion 87 ± 2 %), with the main compounds being pentacosene (24 ± 6 %), heptacosene (31 ± 3 %), nonacosene (44 ± 3 %), and hentriacontene (3 ± 0.8 %), as well as the alkane pentacosane (30 ± 3 %). Among the minor compounds was the alkene octacosene. The extracts of control bees contained 56 substances, of which 32 were identified as alkanes, alkenes, and alkadienes; the remaining 24 compounds were not further identified (Table S2.3). The samples of provisioned bees contained a total of 45 substances, including 27 alkanes, alkenes, and alkadienes, as well as the ketone nonacosen-6-one, and 16 unidentified substances (Table S2.3).

The two minor compounds octacosene and nonacosen-6-one occurred only in extracts of *P. gibbosus* females and provisioned bees, but not in those of control bees (Table S2.3). Moreover, the proportions of the major compounds of *P. gibbosus* females (sum of isomers if more than one

occurred) were significantly higher on provisioned than on control bees (Figure 2.1): heptacosene + heptacosadiene (21 ± 6 % *vs.* 2 ± 5 %; *t* test: t = 5.93, p < 0.0001), nonacosene (25 ± 7 % *vs.* 1 ± 1 %; *t* test: t = 9.4, p < 0.0001), and hentriacontene + hentriacontadiene (3 ± 2 % *vs.* 0.2 ± 0.4 %; *t* test: t = 5.39, p = 0.0001).

Most importantly, provisioned bees carried a considerably larger proportion of unsaturated HCs than controls (55 ± 16 % *vs.* 31 ± 26 %; *t* test: t = 2.3, p = 0.04; Figure 2.2). The total amount of cuticular substances on provisioned bees was, on average, twice as high as on control bees; however, this difference was statistically not significant (8.7 ± 6.5 µg *vs.* 4.1 ± 3.9 µg; Mann–Whitney U test, N provisioned bees = 6, N controls = 9, U = 12, exact p = 0.09).

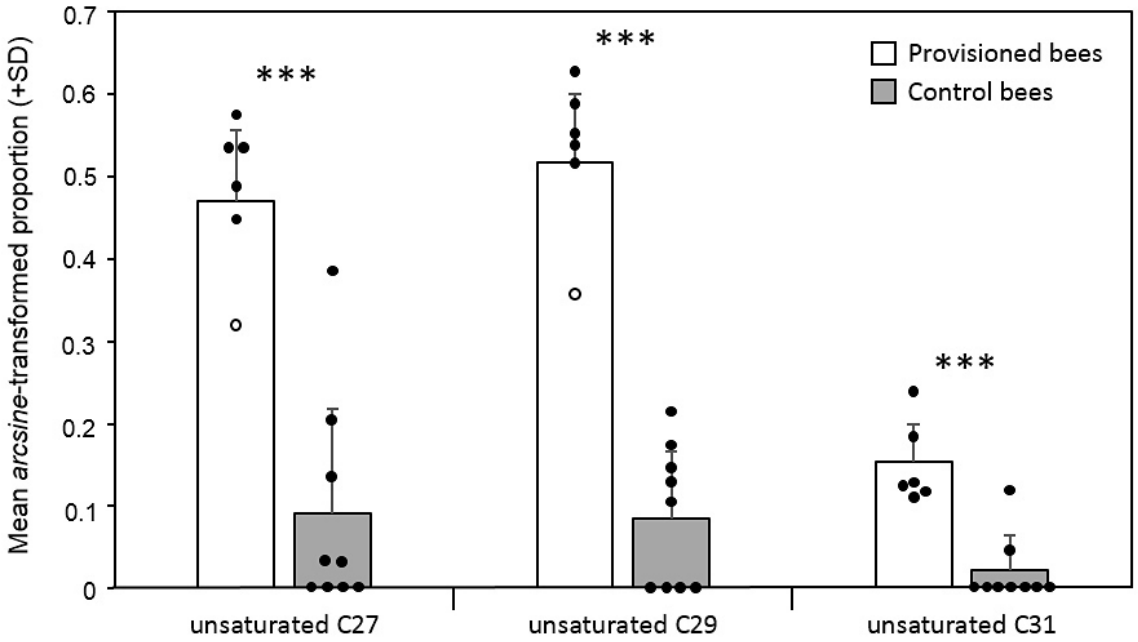


**Figure 2.1** Major hydrocarbons of *P. gibbosus* on provisioned and control bees. Mean arcsine-transformed proportion (+ standard deviation, SD) of heptacosene + heptacosadiene (unsaturated C27), nonacosene (unsaturated C29), and hentriacontene + hentriacontadiene (unsaturated C31) in extracts of provisioned bees taken from *P. gibbosus* brood cells (white bars, N = 6) and control bees which had no contact to the beewolves (grey bars, N = 9); closed circles show individual data points (open circles indicate outliers). Asterisks indicate a significance level of p ≤ 0.001 according to t tests (including outliers).

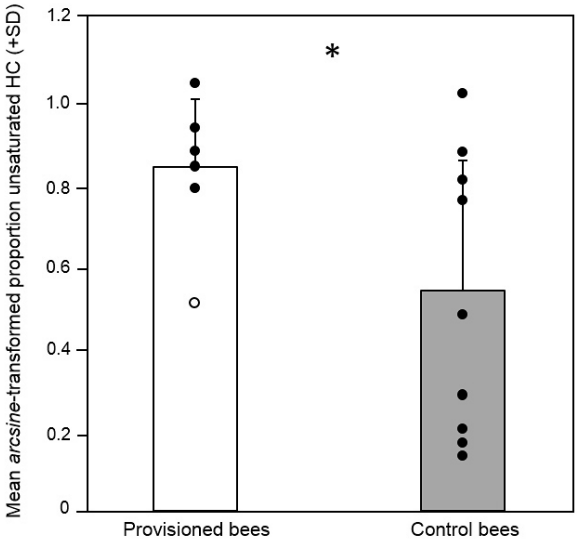


**Figure 2.2** Proportion of unsaturated hydrocarbons on provisioned and control bees. Arcsine-transformed proportion (+ standard deviation, SD) of unsaturated hydrocarbons (HCs) in extracts of provisioned bees (N = 6) taken from *P. gibbosus* brood cells and control bees (N = 9) which had no contact to the beewolves; closed circles show individual data points (open circle indicates outlier). Asterisk indicates a significance level of $p < 0.05$ according to a t test (including outlier).

### 2.4.2 Comparative morphology of head glands

Females of all 26 investigated philanthine species possessed evaginations of the pharynx, originating posterior to the hypopharyngeal plate and, thus, qualifying as PPGs (Figure 2.3, *Philanthus barbiger*). In all species, the wall of the PPG was formed by a monolayered epithelium, whose cells bore hair-like structures on their inner (apical) sides (Figure 2.4). The density of these hairs varied between species (character 6, Table S2.2, Supplementary Material section 2.6.2) and was generally found to decline from the proximal part near the pharynx to the distal parts of the lateral branches of the gland. Besides the paired upper part of the PPG, most species also possessed a smaller sac-like lower part of the gland originating ventrally from the pharynx (character 1, Table S2.2).

The 3D-reconstructions revealed two distinct 'types' of overall PPG morphology: In all Aphilanthopsini (*Aphilanthops frigidus*, *Clypeadon laticinctus*) and Cercerini (*Cerceris arenaria*, *Cerceris quinquefasciata*) each side of both upper and lower part of the PPG consisted of a simple tube-shaped evagination of the pharynx, with the glands of *Aphilanthops* and *Clypeadon* being somewhat stouter than those of *Cerceris* (Figure 2.5 A - D). Except for *A. frigidus*, where the two sides of the upper part of the PPG shared one opening, each PPG-tube had a separate opening to the pharynx (characters 4 and 8, Table S2.2).

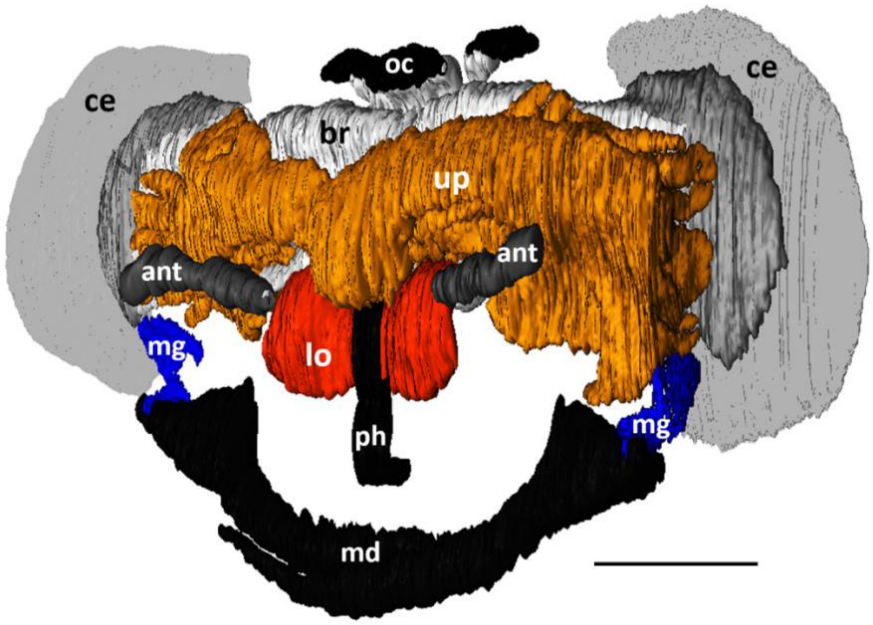


**Figure 2.3** 3D-reconstruction of the head structures of a female *Philanthus barbiger*. The upper reservoir of the PPG (orange) is located in front of the brain (light grey) and extends laterally towards the compound eyes (grey), the sac-like lower reservoir of the gland (red) originates ventrally from the pharynx (black). The small MG reservoirs (blue) are located laterally on both sides of the head capsule, opening at the mandibular base. Abbreviations: ant, antenna; br, brain; ce, compound eye; lo, lower part of PPG; md, mandibles; mg, mandibular gland reservoir; oc, ocelli; ph, pharynx; up, upper part of PPG. Scale bar = 0.5 mm.

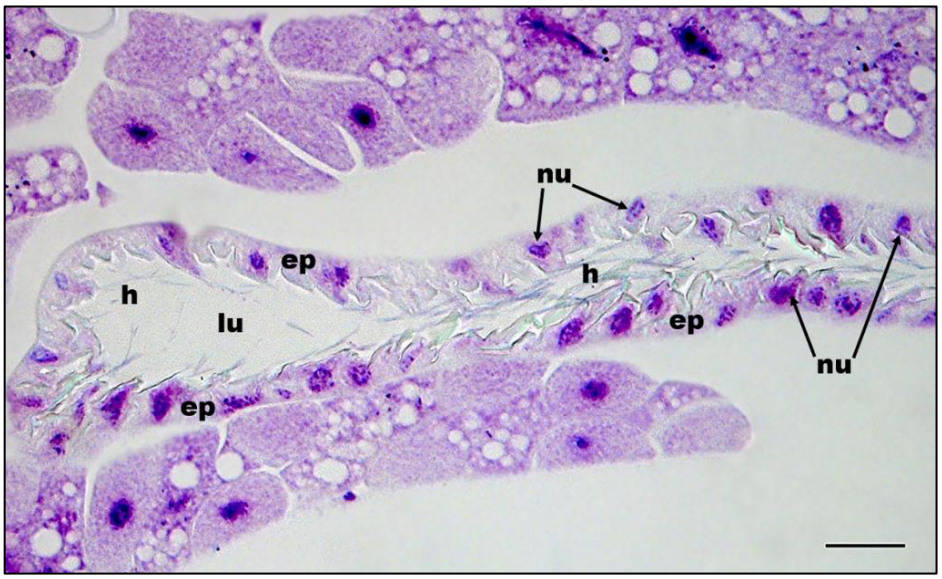


**Figure 2.3** Histological section of the postpharyngeal gland (PPG) of a female *Philanthus rugosus*. The epithelial cells of the upper reservoir of the PPG bear cuticular hairs reaching into the lumen of the gland. Abbreviations: ep, epithelial cells; h, hairs; lu, lumen of the upper PPG reservoir; nu, nuclei of epithelial cells. Scale bar = 25 µm.

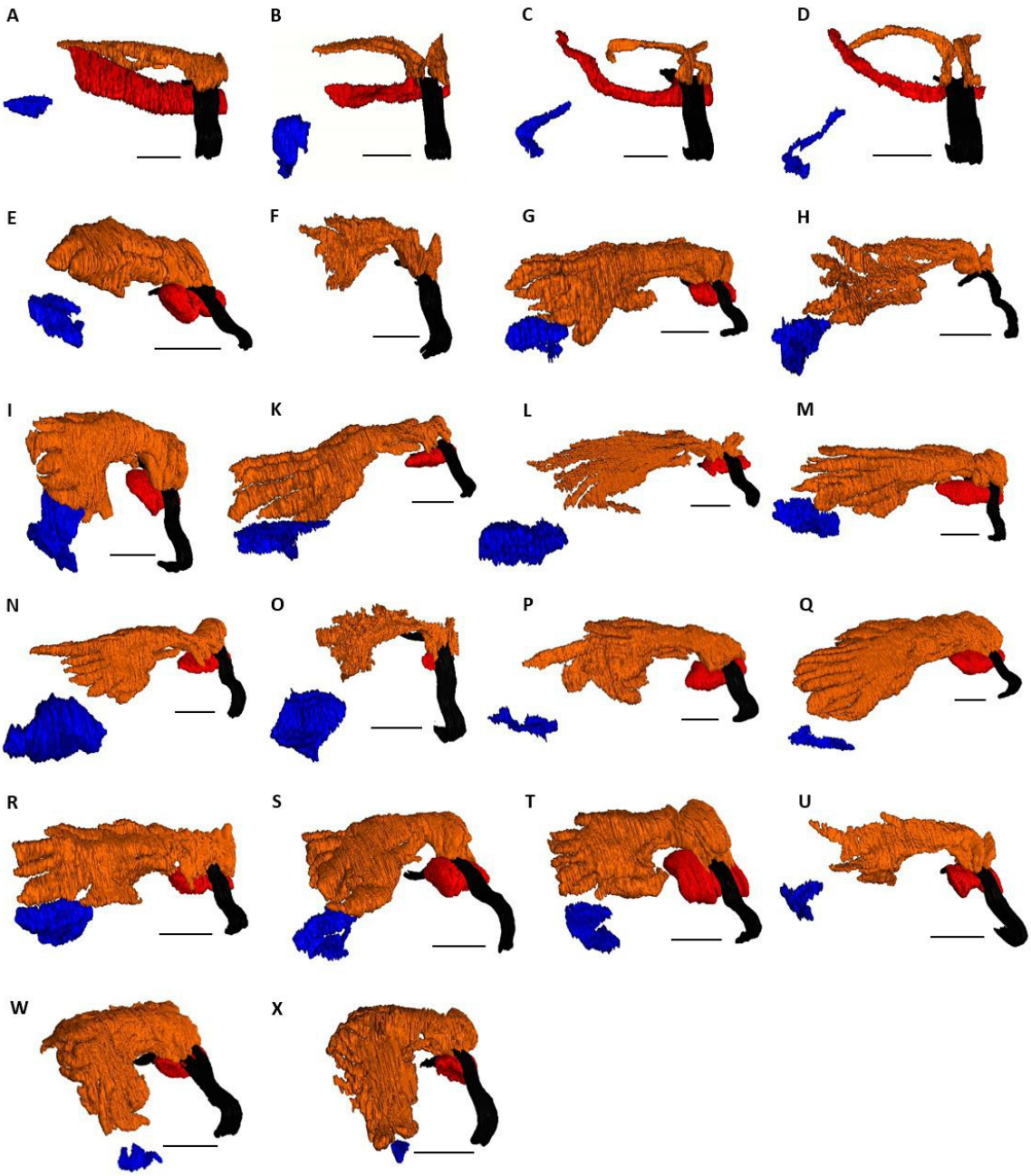


**Figure 2.4** 3D-reconstructions of the head glands of female Philanthinae. The postpharyngeal gland (PPG) is shown in orange and red; the mandibular gland (MG) is shown in blue. Note that for each species only the right part of the head capsule is shown. (A) *Aphilanthops frigidus*, (B) *Clypeadon laticinctus*, (C) *Cerceris arenaria*, (D) *Cerceris quinquefasciata*, (E) *Philanthinus quattuordecimpunctatus*, (F) *Trachypus flavidus* (note that for this species the MG could not be reconstructed based on the available serial histological sections), (G) *Trachypus elongatus*, (H) *Trachypus boharti*, (I) *Philanthus venustus*, (J) *Philanthus t. diadema*, (K) *Philanthus capensis*, (L) *Philanthus loefflingi*, (M) *Philanthus rugosus*, (N) *Philanthus melanderi*, (O) *Philanthus coronatus*, (P) *Philanthus bicinctus*, (Q) *Philanthus ventilabris*, (R) *Philanthus multimaculatus*, (S) *Philanthus barbiger*, (T) *Philanthus gibbosus*, (U) *Philanthus albopilosus*, (V) *Philanthus psyche*. Color code: orange, upper part of the PPG; red, lower part of the PPG; blue, MG; black, pharynx. Scale bars = 0.25 mm.

By contrast, in the 17 investigated species and subspecies of the tribe Philanthini (*Philanthus*, *Trachypus*, and *Philanthinus*) the upper part of the PPG was comparatively larger and showed a glove- or comb-like overall structure with multiple 'fingers' branching off from a common root (Figure 2.5 E - X). The number of these 'fingers' varied among species, ranging from seven to more than 15 per side (character 3, Table S2.2). The left and right part of the upper PPG had either separate openings to the pharynx or shared a common opening (character 4, Table S2.2). The lateral extension of the upper part of the PPG varied among species, ranging from 55 % to 76 % of the head capsule width (character 5, Table S2.2). Two of the investigated *Trachypus* species, *T. flavidus* and *T. boharti*, lacked the lower part of the PPG (Figure 2.5 F and H). In the other species of the Philanthini, the lower part of the PPG consisted of an unpaired saclike evagination that was considerably smaller than the upper part of the gland.

In comparison to the PPG, the differences in the overall morphology of the MG among species were smaller. The MG of all investigated Philanthinae consisted of a pair of lateral and (relative to the PPG) small, unbranched sac-like reservoirs that opened dorsally at the base of the mandibles (Figure 2.3) (characters 9 and 11, Table S2.2). The walls of the MG were formed by a monolayered epithelium, which did not bear any conspicuous surface structures in any species (Figure 2.6) (character 12, Table S2.2). The size of the MG, however, varied somewhat among species (Figure 2.5) (character 10, Table S2.2). In the two investigated *Cerceris* species, the six *Philanthus* species *P. t. triangulum*, *P. t. diadema*, *P. gibbosus*, *Philanthus melanderi*, *Philanthus coronatus*, and *Philanthus albopilosus*, as well as in *T. boharti* and *T. flavidus* the MG reservoir was associated with class 3 gland cells (classification according to Noirot & Quennedey 1974; Figure 2.6), which were identified by their conspicuous end apparatuses and conducting canals. Other cells surrounding the MG in other species might constitute class 1 gland cells (Noirot & Quennedey 1974); however, these cells could not unambiguously be identified as secretory cells with the light-microscopic methods applied.

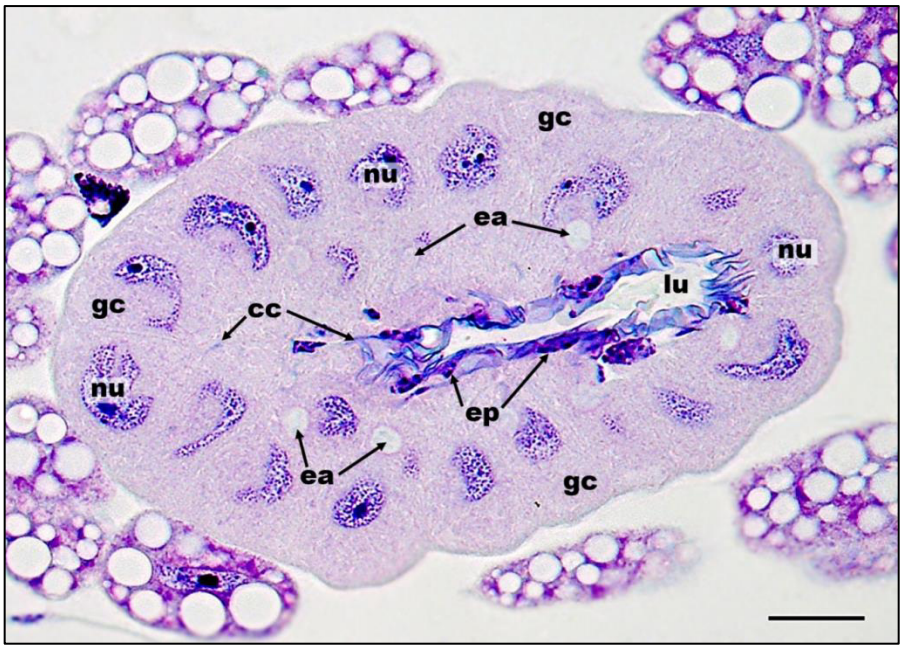


**Figure 2.5** Histological section of the mandibular gland (MG) of a female *Cerceris quinquefasciata*. The class 3 gland cells associated with the MG can be identified by their end apparatuses and conducting canals. Abbreviations: cc, conducting canals of class 3 gland cells; ep, epithelial cells of the MG reservoir; gc, class 3 gland cells; lu, lumen of the MG reservoir; nu, nuclei of class 3 gland cells. Scale bar = 25 µm.

The 13 morphological characters defined for the PPG and MG are summarized in the data matrix displayed in Table S2.2 (Supplementary Material section 2.6.2). A hierarchical cluster analysis using the eight characters defined for the PPG (characters 1-8; Table S2.2, Supplementary Material section 2.6.2) formally confirmed the results obtained by the histological examination and 3D-reconstructions of the PPGs (Figure 2.7). The first bifurcation of the dendrogram separated all species of Philanthini, possessing big glove-shaped PPGs, from both Cercerini and Aphilanthopsini, with simple tube-shaped PPGs. Within the Philanthini, the second bifurcation separated *T. flavidus* and *T. boharti*, which do not possess the lower sac-like evagination of the PPG, from all other Philanthini. The congeneric *T. patagonensis* and *T. elongatus*, however, clustered deeply within the other Philanthini. Generally, no obvious grouping of the species within a tribe could be observed based on PPG morphology. Moreover, most nodes were supported by only low bootstrap values. The inclusion of the data on MG morphology altered the location of single species within the Philanthini, but did not change the basic results obtained by the analysis of the PPG morphology alone (Figure S2.1, Supplementary Material section 2.6.3).

The ancestral state reconstruction showed that the simple tube-shaped PPGs of Cercerini and Aphilanthopsini most likely are the ancestral PPG ‘type’ in the Philanthinae (ML probability: 66 %), while the more complex glove-shaped PPG most likely evolved in the last common ancestor of the tribe Philanthini (ML probability: 96 %) (Figure 2.8).

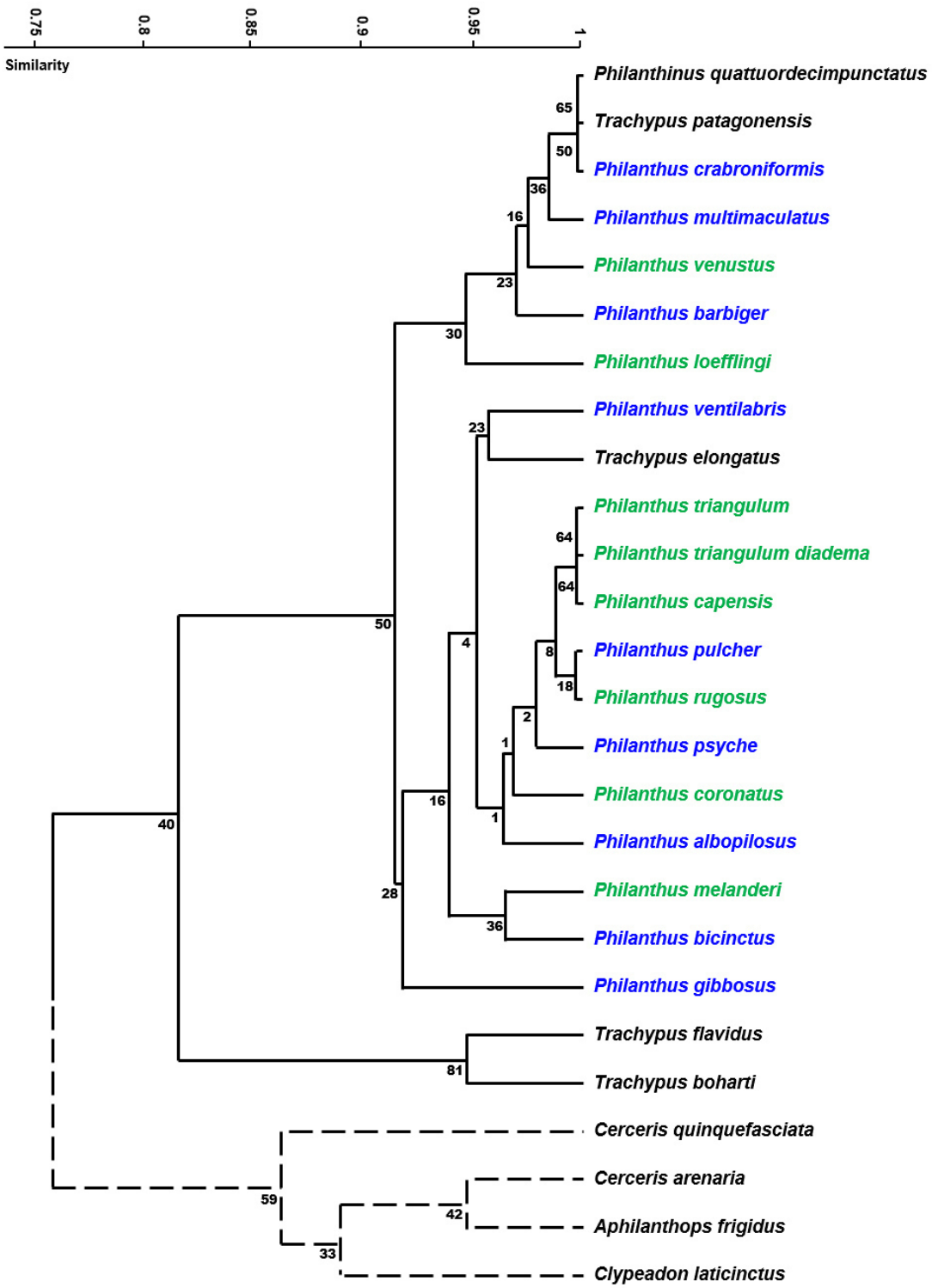


**Figure 2.6** Dendrogram of Philanthinae based on postpharyngeal gland morphology. Dendrogram obtained by a hierarchical cluster analysis based on eight morphological characters of the postpharyngeal gland (PPG) of female Philanthinae. Values at the nodes are bootstrap values. Solid lines indicate members of the tribe Philanthini, dashed lines indicate members of the tribes Cercerini and Aphilanthopsini. Names of European and South African *Philanthus* species are printed in green; names of North American *Philanthus* species are printed in blue. Bray-Curtis was used as similarity index; N bootstrap replicates = 10,000.

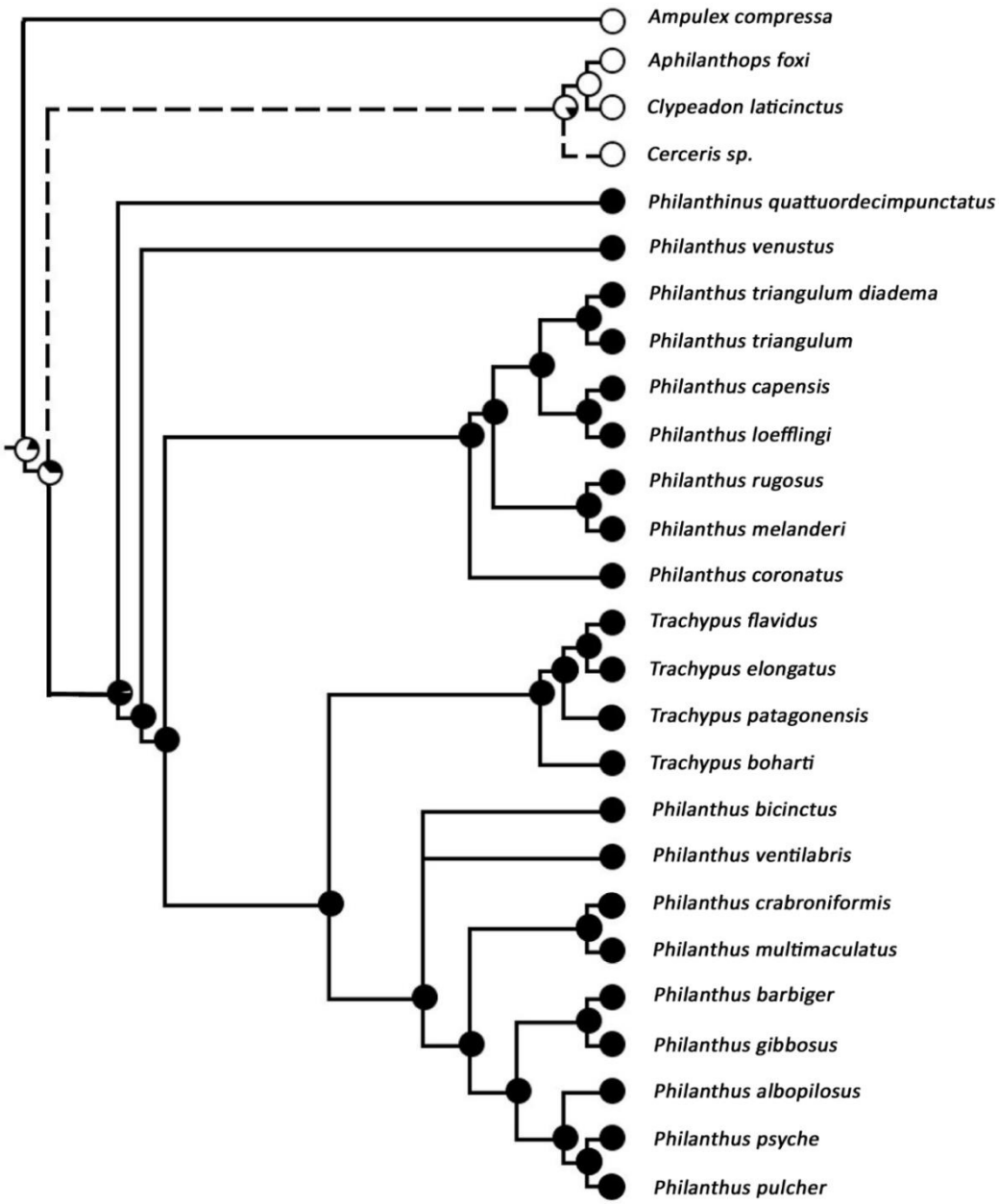


**Figure 2.7** Maximum likelihood (ML) ancestral state reconstruction of the overall shape of the postpharyngeal gland in the Philanthinae. The dendrogram is based on a molecular phylogeny; dashed lines indicate members of the philanthine tribes Cercerini and Aphilanthopsini. ML ancestral state reconstructions, using the Markov k-state 1 parameter model, for each node are visualized using pie charts; color code: white, simple tube-shaped PPGs; black, complex branched PPGs. As both maximum parsimony and ML analyses yielded the same results, only the ML data are shown. Note that the molecular phylogeny comprised *Aphilanthops foxi* and one unidentified *Cerceris* species as representatives of the respective genera.

## 2.5 Discussion

### 2.5.1 Prey embalming in *Philanthus gibbosus*

We provide clear evidence that female *P. gibbosus*, as has been described for *P. triangulum* (Strohm & Linsenmair 2001, Herzner et al. 2007) and *T. elongatus* (Herzner et al. 2013b), embalm their prey with HCs from their PPG: (1) Head extracts of female *P. gibbosus* contained predominately unsaturated HCs, like the PPGs/heads of *P. triangulum* (Strohm et al. 2008), *T. elongatus*, and *T. boharti* (Herzner et al. 2013b), (2) the two components octacosene and nonacosen-6-one occurred only in samples of *P. gibbosus* heads and provisioned bees from their nests, but not on control bees, (3) the proportion of unsaturated HCs was significantly higher on provisioned bees than on control bees, and (4) this increase in the proportion of unsaturated HCs on provisioned bees was mostly due to an increase in the proportion of the major unsaturated HCs of *P. gibbosus* females.

Although the total amount of cuticular HCs was on average twice as high on provisioned bees as on control bees, the difference was not statistically significant. This may be due to the high variance in the total amount of substances within the two groups, which is partly explained by the fact that different halictid species were included in the analysis. The proportion of unsaturated compounds, on the other hand, was significantly higher on provisioned bees than on controls. Similar results (i.e. a significant difference with regard to the proportion of unsaturated HCs but not the total amount of HCs on provisioned *vs.* control bees) have been obtained for *T. elongatus* (Herzner et al. 2013b). In *P. triangulum*, by contrast, the prey embalming results in a significant increase in both the total amount of cuticular HCs and the proportion of unsaturated components (Herzner & Strohm 2007, Herzner et al. 2007). As has been demonstrated in *P. triangulum*, it is the increased proportion of unsaturated HCs, rather than the total amount of cuticular substances, that is crucial for the change in physicochemical properties and the resulting antimicrobial effect of prey embalming (Herzner & Strohm 2007). Thus, we assume that *P. gibbosus* employs a similar mechanism to protect its prey from molding as *P. triangulum* and *T. elongatus*.

According to a recent molecular phylogeny of the Philanthinae (Kaltenpoth et al. 2014), several clades can be distinguished within the tribe Philanthini that generally coincide with their geographical distribution: The genus *Philanthinus* constitutes the most basal taxon of the tribe. The North American *Philanthus* and the South American *Trachypus* are sister taxa (rather than

*Trachypus* being a separate genus, Kaltenpoth et al. 2014) that group within the European and African *Philanthus* species. The fact that the three species that have yet been shown to embalm their prey (the Afro-European *P. triangulum*, the North American *P. gibbosus* and the South American *T. elongatus*) represent the three major clades of the Philanthini suggests that prey embalming is widespread at least within this tribe.

### 2.5.2 Comparative morphology of head glands

All three species that have been shown to employ prey embalming (*P. triangulum*, *P. gibbosus*, and *T. elongatus*) have rather large and complex PPGs. The PPG of female *P. triangulum* contains a mean amount of 330 µg of secretion (maximum: 1,400 µg) (Strohm et al. 2008), of which approximately 80 to 110 µg are applied to a single prey item (Herzner & Strohm 2007, Herzner et al. 2007). Even though the other two species seem to apply less secretion onto their prey, it stands to reason that a large and complex PPG is a prerequisite for prey embalming. The occurrence, morphology, and size of the PPG may hence allow for inferences about the origin and distribution of the prey embalming behavior within the subfamily Philanthinae.

All 26 philanthine species under study, including members of the hitherto not investigated genera *Philanthinus*, *Cerceris*, *Clypeadon*, and *Aphilanthops*, possess head glands that can be classified as PPGs due to their location and basic morphology. As in *P. triangulum* (Strohm et al. 2007), *T. elongatus*, and *T. boharti* (Herzner et al. 2013b), the PPGs of the newly investigated species consist of paired evaginations of the pharynx anterior to the brain, constituting the upper part of the PPG, and an either paired or unpaired lower part (which is missing in some species). In all cases, the gland reservoir is bordered by a monolayered epithelium, the cells of which bear conspicuous hairs that reach into the lumen of the gland.

However, the histological investigation and 3D-reconstructions of the PPGs also revealed distinct differences in the morphology of the PPG between the tribes: The PPGs of all members of the Philanthini (*Philanthus*, *Trachypus*, and *Philanthinus*) are rather uniform in that they possess a voluminous upper part consisting of multiple ‘fingers’ branching off from a common root and the lower part comprising a considerably smaller unpaired sac-like evagination of the pharynx, thus, closely resembling the PPGs of *P. triangulum* (Strohm et al. 2007), as well as *T. elongatus* and *T. boharti* (Herzner et al. 2013b).

The structure of the PPG in females of the Cercerini and Aphilanthopsini differed markedly in that both upper and lower part of the PPG are paired tube-shaped evaginations of the pharynx that do not show any branching. Moreover, the glands are smaller than those found in the Philanthini.

A hierarchical cluster analysis, based on the morphological characters of the PPG, confirmed the described pattern: The first bifurcation separates the Philanthini from both Cercerini and Aphilanthopsini. Within the Philanthini, however, PPG morphology does not mirror the phylogenetic and phylogeographic relationships (e.g. North American *vs.* European/South African *Philanthus* species) within the tribe (Kaltenpoth et al. 2014). Remarkably, the shape of the PPG of female Cercerini/Aphilanthopsini resembles the PPG of the cockroach wasp *A. compressa*, a more basal taxon of the Apoidea (Melo 1999), and the only other apoid wasp for which a PPG has been described (Herzner et al. 2011b). Both sexes of *A. compressa* possess a simple PPG that consists of only one pair of small tubular evaginations of the pharynx and contains mostly HCs (Herzner et al. 2011b). The ancestral state reconstruction of PPG shape using *A. compressa* as outgroup defined the simpler tube-shaped PPGs as the ancestral gland 'type' within the Philanthinae. We therefore conclude that the large complex PPGs evolved after the branching off of the Philanthini, thus representing an autapomorphy of this tribe (Figure 2.9).

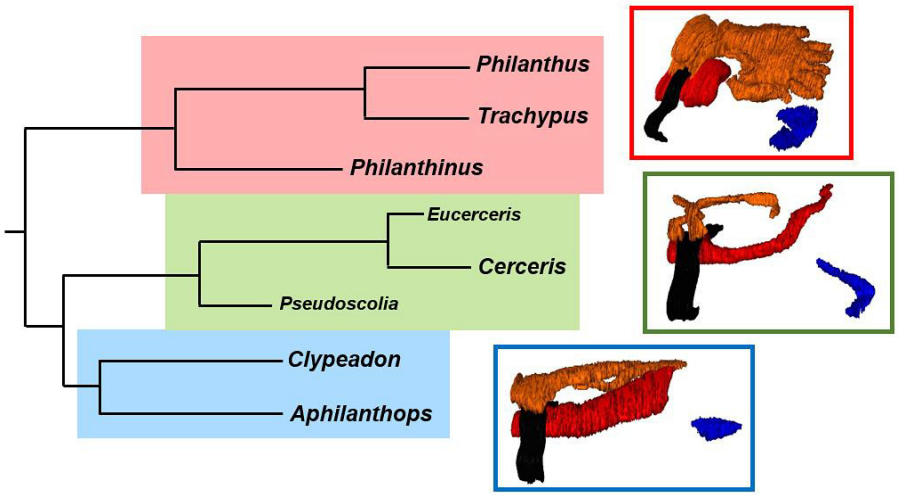


**Figure 2.8** Differences in gland morphology between different tribes of the Philanthinae. The morphology of the postpharyngeal gland (PPG) differs markedly between the three tribes of the subfamily Philanthinae, with all species of the Philanthini (red) possessing complex glove-shaped glands (exemplary 3D-reconstruction: *Philanthus barbiger*), while all investigated species of both Cercerini (green; 3D-reconstruction: *Cerceris arenaria*) and Aphilanthopsini (blue; 3D-reconstruction: *Aphilanthops frigidus*) possess simple tube-shaped glands. Note that for the Cercerini the two genera *Eucerceris* and *Pseudoscolia* (small font) have not been investigated in this study. Dendrogram modified after Alexander (1992).

### 2.5.3 Evolution of prey embalming and complex PPGs

It is tempting to speculate that, corresponding to the observed pattern for PPG size and morphology, the antimicrobial defense mechanism, involving the embalming of the prey with PPG secretion, as described in *P. triangulum* (Herzner & Strohm 2007, Herzner et al. 2007), *P. gibbosus* (this study), and *T. elongatus* (Herzner et al. 2013b), has likewise evolved only in the Philanthini. The fact that both Cercerini and Aphilanthopsini possess only simple and comparatively smaller PPGs suggests that these species do not utilize the same prey preservation strategy as the Philanthini. Consistent with this view, female *A. compressa* do not use their PPGs for embalming their cockroach prey that serves as larval food. The comparatively small PPG of *A. compressa* has been proposed to rather function as a HC storage organ for other purposes (Herzner et al. 2011b).

There may be two possible explanations for this presumptive change in function of the PPG among basal and derived tribes of the Philanthinae. First, the Cercerini and Aphilanthopsini may use one of their two other head glands, the hypopharyngeal gland (which was present in all investigated species) or the MG, for prey embalming. The former, however, is an unlikely candidate as it lacks a reservoir (K. Weiss, *unpublished data*) and because in Hymenoptera its function seems to be restricted to the context of nutrition and digestion (Knecht & Kaatz 1990, Cruz-Landim & Costa 1998, do Amaral & Caetano 2005, Britto & Caetano 2008). The MG of female Philanthinae, whose function is as yet unknown, does comprise a reservoir (Ågren 1977, Evans & O'Neill 1988; this study) and was therefore included in our study. However, our results show that the MGs of female Cercerini and Aphilanthopsini are not larger than in the Philanthini, thus providing no evidence for an enlargement of the MG of the former two tribes to compensate for the smaller volume of their PPGs to allow for prey embalming. Still, as the MG does contain antimicrobial compounds in other Hymenoptera (e.g. Brough 1983, Cane et al. 1983, de Lima Mendoça et al. 2009), a role of this gland in the antimicrobial brood defense of some Philanthinae cannot be ruled out.

Second, the development of a large gland, the production of copious amounts of HCs, and the time consuming embalming of the prey are likely to entail costs (Herzner et al. 2011a). Therefore, prey embalming will only evolve under a selection regime under which its benefits outweigh these costs. The crucial factor in this regard may be the risk of fungal infestations of the larval provisions, which may depend on the microclimate in the nest as well as the susceptibility of the prey to opportunistic mold fungi.

The nest microclimate appears to be rather similar among the Philanthinae (nests in sunny sandy soil), but there are considerable differences in the prey spectrum among the Philanthinae. Members of the Philanthini provision predominantly bees and occasionally other, mostly aculeate Hymenoptera (e.g. Evans & O'Neill 1988). Aphilanthopsini hunt exclusively on ants (Ristich 1956, Evans 1962). Whereas some *Cerceris* species prey upon bees, most Cercerini (including the two investigated in this study) rely on Coleoptera (mainly weevils and buprestids, e.g. Bohart & Menke 1976) as larval provisions. Due to the huge differences in their ecology, these diverse prey taxa may vary in their susceptibility to microbial infestation.

Ants, the prey of the Aphilanthopsini, possess an array of elaborate individual and social immune defenses involving glandular secretions (Brough 1983, de Lima Mendoça et al. 2009) and hygienic behaviors (reviewed in Cremer 2007) that might render them less susceptible to both, entomopathogenic as well as opportunistic fungi. Weevils, the prey of most Cercerini, generally feed on plants that contain secondary compounds like e.g. isoflavones, terpenes, and terpenoids (Zahradník 1985, Phillips & Croteau 1999, Kainulainen & Holopainen 2002, Malinowski & Sierpinska 2005, Hovelstad et al. 2006, Polidori et al. 2007, Saviranta et al. 2008). The sequestration of these compounds, which often show antimicrobial activity (Hovelstad et al. 2006, Saviranta et al. 2008), might provide a certain kind of resistance to microbial attacks.

Larval provisions of the Philanthini, by comparison, might be particularly prone to microbial attack since bees are likely to obtain a range of microorganisms while foraging on flowers e.g. via contaminated pollen (González et al. 2005, Foley et al. 2014) or by transmission from other flower visitors (McArt et al. 2014, Goulson & Hughes 2015). These microorganisms include specific or opportunistic pathogens of Hymenoptera (Foley et al. 2014, Goulson & Hughes 2015, Maxfield-Tylor et al. 2015) and, probably of more importance concerning the infestation risk of larval provisions, Ochratoxin and Aflatoxin producing mold fungi (González et al. 2005, Foley et al. 2014). The view that the Philanthini experience exceptional threats by microbes is supported by the fact that another antimicrobial strategy, the symbiosis with antibiotics producing Streptomyces bacteria (Kaltenpoth et al. 2005, Kroiss et al. 2010, Koehler et al. 2013), is also restricted to the tribe Philanthini (Kaltenpoth et al. 2006, 2010, 2012, 2014).

Assuming that bees are a 'riskier' food resource than beetles and ants, it would be compelling to further investigate the Aphilanthopsini and in particular the genus *Cerceris* for the morphology of the PPG and the implementation of prey embalming. One might expect bee-hunting *Cerceris* to

exhibit some kind of prey preservation behavior similar to the Philanthini, and thus also to possess larger and more complex PPGs than their beetle-hunting congeners.

### 2.5.4 Conclusions

All investigated species of the tribe Philanthini possess complex and large PPGs, whereas the investigated Aphilanthopsini and Cercerini bear comparably simple and smaller PPGs. Based on our findings it seems likely that all Philanthini employ prey embalming in a similar way as described for *P. triangulum* (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2007), *P. gibbosus* (this study), and *T. elongatus* (Herzner et al. 2013b). In *P. triangulum*, prey embalming has significant positive effects on the females' reproductive success by enhancing offspring survival (Herzner et al. 2011a). However, the maintenance of a complex PPG and the production of high amounts of HCs for prey embalming is likely to entail costs for the female (Herzner et al. 2011a). The tribe Philanthini seems to have experienced stronger selection for effective prey preservation than most species of the Aphilanthopsini and Cercerini, owing to the presumably higher susceptibility to fungal infestations of the prey taxa used as larval food, so that the fitness gains eventually outweighed the costs involved in prey embalming. Future studies on the PPG and prey preservation in the Philanthinae will shed further light on the evolution of this gland and the intriguing parental care behavior of prey embalming. Our results show that the subfamily Philanthinae provides an excellent example of how even minor differences in ecology may influence the evolution of hygienic behaviors and the related morphological characters. Given the huge diversity of mass-provisioning wasps (pompilids, ampulicids, sphecids, crabronids, eumenids; O`Neill 2001, Bohart & Menke 1976) and their respective nesting sites and prey taxa, there are probably many more elaborate strategies of larval food preservation to be discovered.

## 2.6 Supplementary material

### 2.6.1 Prey embalming in *Philanthus gibbosus*

#### *2.6.1.1 Specimens and rearing conditions*

Female *P. gibbosus* were collected on flowers in Madison (Wisconsin, USA) on August 15th and 16th, 2009, and transported alive to the University of Regensburg (Bavaria, Germany). The beewolves were kept in observation cages as described earlier for *P. triangulum* (Strohm & Linsenmair 1994), but because of the smaller size of *P. gibbosus*, the thickness of the sand layer in the nesting compartment was reduced to about 6 mm. The female *P. gibbosus* were supplied *ad libitum* with honey and halictid bees (Hymenoptera, Halictidae) that were collected daily in the Botanical Garden of the University of Regensburg. In order to assess whether *P. gibbosus* females embalm their prey with hydrocarbons (HCs) from their postpharyngeal gland (PPG), as has previously been described for *P. triangulum* (Herzner & Strohm 2007, Herzner et al. 2007) and *T. elongatus* (Herzner et al. 2013b), six paralyzed bees were removed from two artificial *P. gibbosus* brood cells (three bees each; hereafter referred to as 'provisioned bees') for chemical analysis. For comparison, nine halictid bees were collected in the field and analyzed without prior contact to *P. gibbosus* (hereafter referred to as 'control bees'). Additionally, heads of four *P. gibbosus* females that had been collected in Salt Lake City and kindly provided by Jon Seger (University of Utah) were extracted for analysis of their HC composition. The analysis of whole heads yields similar results as the analysis of dissected PPG reservoirs since the PPG contains comparatively huge amounts of HCs and their composition is virtually identical to that of the cuticle (Herzner et al. 2013b, Strohm et al. 2008).

#### *2.6.1.2 Molecular identification of prey species*

To reliably identify the halictid bee species, we sequenced a portion of the nuclear long-wavelength rhodopsin (*lwrh*) and the mitochondrial cytochrome oxidase I (*coxI*) gene, respectively (Table S2.1). Briefly, after hexane extraction of samples for chemical analysis, DNA was extracted from the thorax using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA) according to the manufacturer's instructions. The DNA pellet was resuspended in 50 µl low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA). PCRs were performed on a VWR thermocycler in total reaction volumes of 12.5 µl containing 1 µl of template, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 240 µM deoxynucleoside

triphosphates, 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (VWR). The primer pairs LWRHfor1 (5'-AATTGCTATTAYGARACNTGGGT-3')/LWRHrev1 (5'-ATATGGAGTCCANGCCATRAACC A-3') and Jerry (5'-CAACATTTATTTTGATTTTTTGG-3') / Pat (5'-TCCAATGCACTAATCTGCCATATTA-3') were used for amplification of *lwrh* and *coxI*, respectively. Cycle parameters were as follows: 3 min at 94°C, followed by 35-38 cycles of 94°C for 40 s, a primer-specific annealing temperature for 40 s (58.5°C for *lwrh* and 51°C for *coxI*), and 72°C for 40 s, and a final extension time of 4 min at 72°C. Unidirectional sequencing with primers LWRHfor1 and Jerry, respectively, was done on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, USA) in the Department of Entomology at the Max Planck Institute for Chemical Ecology (Jena, Germany). Curated sequences were compared with the NCBI database using BLASTn (Altschul et al. 2009).

**Table S2.1** Bee species included in the chemical analysis. Halictid species taken from *P. gibbosus* brood cells (provisioned bees) and field-caught bees that had no contact to the beewolves (control bees) according to a comparison with the NCBI database using BLASTn (Blast identity). Abbreviations: lwrh, nuclear long-wavelength rhodopsin gene; coxI, mitochondrial cytochrome oxidase I gene.

| Group | Species | Blast identity | Gene |
|---|---|---|---|
| **Provisioned bees** | *Lasioglossum villosulum* | 98 % | *lwrh* |
| | *Halictus kessleri* | 96 % | *lwrh* |
| | *Halictus tumulorum* | 97 % | *lwrh* |
| | *Halictus simplex* | 97 % | *lwrh* |
| | *Halictus tumulorum* | 97 % | *lwrh* |
| | *Halictus tumulorum* | 98 % | *lwrh* |
| **Control bees** | *Halictus pseudomaculatus* | 97 % | *lwrh* |
| | *Halictus kessleri* | 97 % | *lwrh* |
| | *Halictus kessleri* | 96 % | *lwrh* |
| | *Lasioglossum villosulum* | 99 % | *lwrh* |
| | not identified | --- | --- |
| | *Halictus kessleri* | 96 % | *lwrh* |
| | *Halictus tumulorum* | 99 % | *coxI* |
| | *Halictus kessleri* | 97 % | *lwrh* |
| | *Halictus kessleri* | 96 % | *lwrh* |
| | *Halictus kessleri* | 96 % | *lwrh* |
| | *Halictus scabiosae* | 97 % | *lwrh* |

*2.6.1.3 Chemical analysis*

Specimens were extracted for 10 minutes in approximately 1 ml of hexane. For the bee samples 2 µg of octadecane were added as an internal standard, to allow the quantification of the absolute amounts of the compounds. For each sample, the solvent was evaporated under a gentle stream of nitrogen, then 50-100 µl hexane were added, and the extract was transferred to a 200 µl GC-µ-vial (CZT, Kriftel, Germany). An aliquot of 1 µl of each sample was injected into an Agilent 6890N

Series GC system coupled to an Agilent 5973 insert mass selective detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a nonpolar RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df = 0.25 µm; Capital Analytical Ltd., Leeds, UK; temperature program: from 60°C to 300°C at 5°C/min and held for 1 min at 60°C and for 10 min at 300°C). Helium was used as the carrier gas, with a constant flow of 1 ml/min. A split/splitless injector was operated at 250°C in the splitless mode (60s). Electron impact mass spectra were recorded with an ionization voltage of 70 eV, a source temperature of 230°C, and an interface temperature of 315°C. The software MSD ChemStation for Windows was used for data acquisition. *N*-Alkanes were identified by the comparison of their retention times and mass spectra to those of synthetic reference substances. Linear retention indices (LRIs) for all other substances were calculated according to Van den Dool & Kratz (1963) and alkenes were identified by their LRIs and mass spectra as described in Strohm et al. (2008). The structure of the unsaturated ketone nonacosen-6-one was tentatively assigned by its mass spectrum as described previously (Herzner et al. 2013b).

*2.6.1.4 Data analysis*

Besides the peaks that were identified as alkanes, alkenes, and alkadienes, we detected 29 additional substances in the extracts of halictid bees, none of which occurred in *P. gibbosus* samples (with the exception of one putative hexacosene, see Table S2.2). Since the aim of this investigation was to assess whether *P. gibbosus* females apply substances to the surface of their prey, no further efforts were made to identify these substances, occurring only in bee samples. As the peaks of the different isomers of the alkenes in some cases were not completely separated in the GC profile, the peak areas of all isomers of a given chain length were generally combined for further analysis. The total amounts of substances for each individual sample of provisioned and control bees were calculated using the internal standard. Since the *P. gibbosus* samples had been analyzed without internal standard, absolute amounts were not calculated for this group. Additionally, the total peak area was standardized to 100% and the relative amounts of substances were calculated for each individual sample from all three groups. Inspection of the chromatograms revealed considerable differences between samples of the three groups in the peak areas of the three alkenes dominating the GC profile of female *P. gibbosus*, namely heptacosene, nonacosene, and hentriacontene. Therefore, the proportions of each of these substances were compared between provisioned and control bees. To assess whether provisioned bees carried larger amounts of HCs and a higher proportion of unsaturated HCs, we compared the total amounts of cuticular substances, as well as the relative amounts of unsaturated

hydrocarbons between provisioned bees and control bees. All relative values were *arcsine*-transformed prior to analysis. All statistical comparisons were conducted with *t* tests (using test statistics for equal or unequal variances, respectively, depending on the results of preceding Levene's tests for homogeneity of variance), using the statistics software package PAST (Version 2.08b) (Hammer et al. 2001).

### 2.6.2 Comparative morphology of head glands: Coding of character states

As the result of a comprehensive examination of both semithin histological sections and 3D-reconstructions of the head glands of female Philanthinae, we defined 13 morphological characters for the comparative analysis of the PPG and MG of the 26 philanthine species under study (Table 2.1, main text). For each character, different character states were categorized and numerically coded for the statistical analysis (see below; numbers in parentheses). Characters 1, 2, 7, 9, 11, and 13 are coded non-additive as their different states are considered equivalent; therefore, for these characters the numerical code does not imply a quantitative ranking of their states. Note that the two characters defined for the lower part of the PPG reservoir (characters 7 and 8) could only be defined if this part of the reservoir was present [i.e. character 1 in state (0)].

**PPG:**

1. O*verall structure of the PPG*.
    - **(0)** PPG consists of an upper reservoir originating from the dorsal side of the pharynx, and a lower evagination originating ventrally from the pharynx
    - **(1)** PPG consists of only the upper reservoir
2. *Shape of the upper PPG*.
    - **(0)** simple tube-shaped evagination
    - **(1)** comb-shaped: the complex upper PPG consists of multiple lobes originating from a common root with all branches being oriented to one side
    - **(2)** glove-shaped: the complex upper PPG consists of multiple 'fingers' originating from the common root of the upper PPG
    - **(3)** the complex upper PPG consists of multiple lobes originating from a common root but its form lies somewhere between (1) and (2)

3. *Number of lobes of the upper PPG (per side)*.

**(0)** 1

**(1)** < 10

**(2)** 10 - 15

**(3)** > 15

4. *Number of openings of the upper part of the PPG to the pharynx*.

**(0)** right and left part of the lateral symmetric upper PPG reservoir share one opening to the pharynx

**(1)** both right and left part of the upper PPG each have a separate opening to the pharynx

5. *Relative lateral extension of the upper PPG*. In order to exclude size effects, the lateral extension of the upper PPG was calculated in relation to the head capsule width as a measure of relative gland size. This was estimated as the proportion of the total number of semithin sections of the head capsule including compound eyes that showed structures belonging to the PPG.

**(0)** 45 % or less

**(1)** 46 - 50 %

**(2)** 51 - 55 %

**(3)** 56 - 60 %

**(4)** 61 - 65 %

**(5)** 66 - 70 %

**(6)** 71 - 75 %

**(7)** > 75 %

6. *Inner walls of the PPG*. The epithelial cells of the PPG reservoir can bear hairs on their inner side, reaching into the lumen of the gland. The density of these hairs can vary between the proximal part, i.e. near the opening to the pharynx, and the distal parts of the gland, extending toward the compound eyes.

**(0)** no hairs

**(1)** few unevenly distributed hairs: few hairs near the opening to the pharynx but no hairs in the distal parts of the lobes

**(2)** few but evenly distributed hairs throughout the gland

**(3)** many unevenly distributed hairs: many hairs near the opening to the pharynx but only few hairs in the distal parts of the lobes

**(4)** many hairs, evenly distributed throughout the gland

7. *Shape of the lower PPG*.

**(0)** unpaired sac-shaped evagination of the pharynx

**(1)** paired tube-shaped evagination of the pharynx

8. *Number of openings of the lower part of the PPG to the pharynx*.

**(0)** right and left part of the lower PPG share one opening to the pharynx

**(1)** right and left part of the lower PPG each have a separate opening to the pharynx

**MG:**

9. *Structure of the MG*. Similar to the PPG, the MG can be formed by an upper and a lower reservoir, opening dorsally and ventrally to the mandible, respectively.

**(0)** only the lower part of the MG is developed

**(1)** only the upper part of the MG is developed

**(2)** both parts of the MG are developed

10. *MG size*. The volume of the MG depends on the filling status of the gland reservoir. Therefore, its size was assessed as the extension of the MG reservoir into the head capsule based on semithin histological sections and 3D-reconstructions, rather than by measuring its volume.

**(0)** < 1/4 of head capsule

**(1)** about 1/4 of head capsule

**(2)** 1/4 - 1/2 of head capsule

11. *Branching of the MG*.

**(0)** unbranched: the MG reservoir consists of one sac-like evagination

**(1)** branched: the MG reservoir consists of distinct branches

12. *Inner walls of the MG*. As with the PPG, the epithelial cells of the MG reservoir can bear hairs on their inner side, reaching into the lumen of the gland.

**(0)** no hairs

**(1)** few hairs

**(2)** many hairs

13. *Gland cells associated with the MG*. Typically the MG of Hymenoptera is associated with class 3 gland cells (classified according to Noirot & Quennedey 1974), that can be identified by the occurrence of end apparatuses and conducting canals. However, in some of the investigated species there are no class 3 gland cells associated with the MG; however, there are cells that may be classified as class 1 gland cells, but these cells could not unambiguously be identified as secretory cells with the applied light-microscopic methods. Hence, we distinguished only cases with class 3 gland cells and those without these.

**(0)** the MG is not associated with class 3 gland cells

**(1)** the MG is associated with class 3 gland cells

**Table S2.2** Character matrix for the comparative statistical analysis of head gland morphology of female Philanthinae. The numbering of the morphological characters of the PPG and the MG and the numeric coding of the character states correspond to the description in the Supplementary Material section 2.6.2 above. ?, character state could not be determined; ---, character not present in this species.

| | PPG characters | | | | | | | | MG characters | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Taxon** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
| ***Aphilanthops frigidus*** | 0 | 0 | 0 | 0 | 4 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| ***Clypeadon laticinctus*** | 0 | 0 | 0 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| ***Cerceris arenaria*** | 0 | 0 | 0 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| ***Cerceris quinquefasciata*** | 0 | 0 | 0 | 1 | 0 | 4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| ***Philanthinus quattuordecimpunctatus*** | 0 | 2 | 1 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Trachypus flavidus*** | 1 | 2 | 2 | ? | 6 | 3 | --- | --- | ? | ? | ? | ? | 1 |
| ***Trachypus elongatus*** | 0 | 2 | 2 | 0 | 6 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Trachypus patagonensis*** | 0 | ? | ? | ? | ? | ? | 0 | ? | 1 | 1 | 0 | 0 | 0 |
| ***Trachypus boharti*** | 1 | 1 | 2 | 1 | 7 | 3 | --- | --- | 1 | 1 | 0 | 0 | 1 |
| ***Philanthus venustus*** | 0 | 2 | 2 | 0 | 4 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus triangulum triangulum*** | 0 | 2 | ? | 1 | ? | 3 | 0 | 0 | 1 | 2 | 0 | 0 | 1 |
| ***Philanthus triangulum diadema*** | 0 | 2 | 2 | 1 | 5 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| ***Philanthus capensis*** | 0 | 2 | 2 | 1 | 5 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus loefflingi*** | 0 | 2 | 2 | 1 | 6 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus rugosus*** | 0 | 2 | 2 | 1 | 4 | 3 | 0 | 0 | 1 | 2 | 0 | 0 | 0 |
| ***Philanthus melanderi*** | 0 | 3 | 2 | 1 | 3 | 3 | 0 | 0 | 1 | 2 | 0 | 0 | 1 |
| ***Philanthus coronatus*** | 0 | 2 | 2 | 1 | 7 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| ***Philanthus bicinctus*** | 0 | 3 | 3 | 1 | ? | ? | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| ***Philanthus ventilabris*** | 0 | 2 | 2 | 0 | 4 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus crabroniformis*** | 0 | ? | ? | ? | ? | ? | 0 | ? | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus multimaculatus*** | 0 | 2 | 2 | 0 | ? | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus barbiger*** | 0 | 2 | 2 | 0 | 5 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ***Philanthus gibbosus*** | 0 | 2 | 1 | 1 | 2 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| ***Philanthus albopilosus*** | 0 | 3 | 2 | 1 | 5 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| ***Philanthus psyche*** | 0 | 2 | 3 | ? | ? | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| ***Philanthus pulcher*** | 0 | ? | ? | 1 | ? | 3 | 0 | 0 | 1 | ? | ? | 0 | 0 |

## 2.6.3 Supplementary table and figure

**Table S2.3** Chemical composition of the cuticular extracts of *P. gibbosus* females, provisioned bees, and control bees. Abbreviations: P.gib, *P. gibbosus* female heads; PB, provisioned bees; CB, control bees; LRI, linear retention index (calculated in relation to n-alkanes). +, substance detected in at least one sample of the group; -, substance not detected. Substances shaded in grey only occurred in samples of *P. gibbosus* heads and on provisioned bees taken from P. gibbosus brood cells, but not on control bees.

| Substance | LRI | P. gib | PB | CB | Diagnostic ions |
|---|---|---|---|---|---|
| Unidentified 01 | 2052 | - | + | + | |
| **Heneicosadiene 1** | **2069** | **-** | **+** | **+** | **292** |
| **Heneicosadiene 2** | **2081** | **-** | **+** | **+** | **294** |
| ***n*-Heneicosane** | **2100** | **-** | **+** | **+** | **296** |
| Unidentified 02 | 2138 | - | + | + | |
| Unidentified 03 | 2148 | - | - | + | |
| Unidentified 04 | 2166 | - | - | + | |
| Unidentified 05 | 2175 | - | - | + | |
| Unidentified 06 | 2194 | - | - | + | |
| Unidentified 07 | 2201 | - | - | + | |
| Unidentified 08 | 2238 | - | - | + | |
| Unidentified 09 | 2248 | - | + | + | |
| **Tricosadiene** | **2251** | **-** | **-** | **+** | **320** |
| Unidentified 10 | 2259 | - | + | - | |
| **Tricosene 1** | **2274** | **-** | **+** | **+** | **322** |
| **Tricosene 2** | **2281** | **-** | **+** | **+** | **322** |
| Unidentified 11 | 2288 | - | - | + | |
| ***n*-Tricosane** | **2300** | **+** | **+** | **+** | **324** |
| Unidentified 12 | 2338 | - | + | - | |
| Unidentified 13 | 2341 | - | + | + | |
| Unidentified 14 | 2351 | - | + | + | |
| **Tetracosene** | **2370** | **-** | **-** | **+** | **334** |
| Unidentified 15 | 2394 | - | - | + | |
| ***n*-Tetracosane** | **2402** | **+** | **+** | **+** | **338** |
| Unidentified 16 | 2428 | - | - | + | |
| Unidentified 17 | 2433 | - | + | + | |
| Unidentified 18 | 2446 | - | + | + | |
| Unidentified 19 | 2460 | - | + | + | |
| **Pentacosadiene** | **2470** | **+** | **+** | **+** | **348** |
| **Pentacosene 1** | **2475** | **+** | **+** | **+** | **350** |
| **Pentacosene 2** | **2482** | **+** | **+** | **+** | **350** |
| **Pentacosene 3** | **2493** | **+** | **+** | **+** | **350** |
| ***n*-Pentacosane** | **2500** | **+** | **+** | **+** | **352** |
| Unidentified 20 | 2542 | - | + | + | |
| Unidentified 21 | 2553 | - | + | + | |
| **Hexacosene 1** | **2577** | **+** | **-** | **-** | **364** |
| Unidentified 22 [1] | **2584** | **+** | **+** | **-** | |
| Unidentified 23 | 2594 | - | - | + | |
| ***n*-Hexacosane** | **2602** | **-** | **+** | **+** | **366** |
| Unidentified 24 | 2662 | - | + | + | |
| **Heptacosene 1** | **2672** | **-** | **-** | **+** | **378** |
| **Heptacosadiene + Heptacosene 2** | **2676** | **+** | **+** | **+** | **376, 378** |
| **Heptacosene 3** | **2684** | **+** | **+** | **+** | **378** |
| **Heptacosene 4** | **2694** | **+** | **+** | **+** | **378** |
| ***n*-Heptacosane** | **2700** | **+** | **+** | **+** | **380** |
| Unidentified 25 | 2743 | - | + | + | |

**Table S2.3** *continued*

| Substance | LRI | P. gib | PB | CB | Diagnostic ions |
|---|---|---|---|---|---|
| Unidentified 26 | 2755 | - | + | - | |
| **Octacosene** | **2777** | **+** | **+** | **-** | **392** |
| **Nonacosene 1** | **2859** | **-** | **+** | **+** | **406** |
| **Nonacosene 2** | **2865** | **-** | **+** | **+** | **406** |
| **Nonacosene 3** | **2878** | **+** | **+** | **+** | **406** |
| **Nonacosene 4** | **2886** | **+** | **+** | **+** | **406** |
| ***n*-Nonacosane** | **2900** | **+** | **+** | **+** | **408** |
| **Hentriacontadiene 1** | **3019** | **-** | **-** | **+** | **432** |
| **Hentriacontadiene 2** | **3026** | **-** | **-** | **+** | **432** |
| **Hentriacontadiene 3** | **3044** | **-** | **-** | **+** | **432** |
| **Hentriacontadiene 4** | **3051** | **-** | **+** | **+** | **432** |
| **Nonacosen-6-one** | **3060** | **+** | **+** | **-** | **99, 349, 420** |
| **Hentriacontene 1** | **3067** | **+** | **+** | **+** | **434** |
| **Hentriacontene 2** | **3075** | **-** | **+** | **-** | **434** |
| Unidentified 27 | 3087 | - | + | + | |
| Unidentified 28 | 3146 | - | - | + | |
| **Tritriacontadiene 1** | **3192** | **-** | **-** | **+** | **462** |
| **Tritriacontadiene 2** | **3222** | **-** | **+** | **-** | **462** |
| **Tritriacontadiene 3** | **3231** | **-** | **-** | **+** | **462** |

[1] Based on the available partial mass spectrum and the comparison of its LRI to published data, this very minor compound, that occurred only in samples of *P. gibbosus* females and provisioned bees, most likely is a hexacosene. However, it could not unambiguously be identified due to the lack of the molecular ion in the mass spectrum and consequently is classified as unidentified. It is therefore not discussed in the main text.

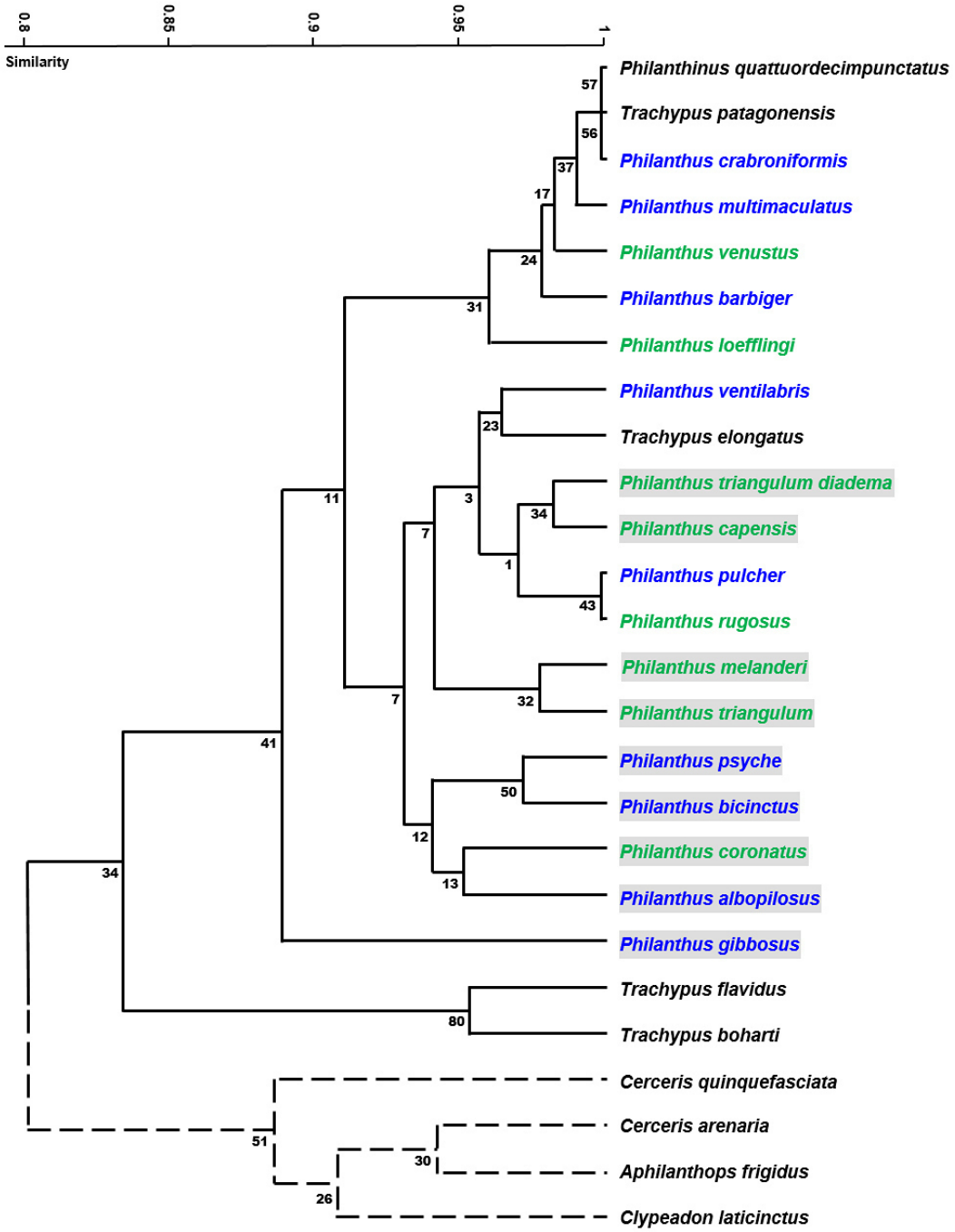


**Figure S2.1** Clustering of species based on PPG and MG morphology. Dendrogram based on the hierarchical cluster analysis of all 13 morphological characters defined for the PPG and MG of female Philanthinae. Values at the nodes are bootstrap values. Solid lines indicate members of the tribe Philanthini, dashed lines indicate members of the tribes Cercerini and Aphilanthopsini. Names of European and South African *Philanthus* species are printed bold; names of North American *Philanthus* species are printed in italics. The grouping of species shaded in gray differs from the cluster analysis of only the eight morphological characters of the PPG (Figure 2.7). Bray-Curtis was used as a similarity measure; N bootstrap replicates = 10,000.

## 2.7 References


Adam H, Czihak G: Arbeitsmethoden der makroskopischen und mikroskopischen Anatomie. Stuttgart: Gustav Fischer Verlag; 1964.

Ågren L: Mandibular gland morphology of *Cerceris rybyensis* (L), (Hymenoptera: Philanthidae). *Zoon* 1977, 5(2):91-95.

Alexander BA: A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic entomology* 1992, 17(2):91-108.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of Molecular Biology* 2009, 215:403-410.

Bohart RM, Menke AS: Sphecid wasps of the world: A generic revision: University of California Press; 1976.

Britto F, Caetano F: Ultrastructural features of the hypopharyngeal glands in the social wasp Polistes versicolor (Hymenoptera: Vespidae). *Insect Science* 2008, 15(3):277-284.

Brough EJ: The antimicrobial activity of the mandibular gland secretion of a formicine ant, *Calomyrmex sp.* (Hymenoptera, Formicidae). *Journal of Invertebrate Pathology* 1983, 42(3):306-311.

Burkepile DE, Parker JD, Woodson CB, Mills HJ, Kubanek J, Sobecky PA, Hay ME: Chemically mediated competition between microbes and animals: Microbes as consumers in food webs. *Ecology* 2006, 87(11):2821-2831.

Cane JH, Gerdin S, Wife G: Mandibular gland secretions of solitary bees (Hymenoptera, Apoidea) - Potential for nest cell disinfection. *Journal of the Kansas Entomological Society* 1983, 56(2):199-204.

Cardona A, Saalfeld S, Schindelin J, Arganda-Carreras I, Preibisch S, Longair M, Tomancak P, Hartenstein V, Douglas RJ: TrakEM2 software for neural circuit reconstruction. *Plos One* 2012, 7(6):e38011.

De Lima Mendonça AD, da Silva CE, de Mesquita FLT, da Silva Campos R, Do Nascimento RR, Ximenes ECPA, Sant'Ana AEG: Antimicrobial activities of components of the glandular secretions of leaf cutting ants of the genus *Atta*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2009, 95(4):295-303.

Do Amaral JBd, Caetano FH: The hypopharyngeal gland of leaf-cutting ants (*Atta sexdens rubropilosa*) (Hymenoptera: Formicidae). *Sociobiology* 2005, 46(3):515-524.

Cremer S, Armitage SAO, Schmid-Hempel P: Social immunity. *Current Biology* 2007, 17(16):R693-R702.

Cruz-Landim Cd, Costa R: Structure and function of the hypopharyngeal glands of Hymenoptera: A comparative approach. *J Comp Biol* 1998, 3(2):151-163.

Eelen D, Borgesen L, Billen J: Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 2006, 87(2):101-111.

Evans HE: A review of nesting behavior of digger wasps of the genus *Aphilanthops*, with special attention to the mechanism of prey carriage. *Behaviour* 1962, 19:239 - 260.

Evans HE, O' Neill KM: The natural history and behavior of North American beewolves. Ithaca: Cornell University Press; 1988.

Foley K, Fazio G, Jensen AB, Hughes WO: The distribution of *Aspergillus spp.* opportunistic parasites in hives and their pathogenicity to honey bees. *Veterinary microbiology* 2014, 169(3):203-210.

Führer E, Willers D: The anal secretions of the endoparasitic larva *Pimpla turionellae*: Sites of production and effects. *Journal of Insect Physiology* 1986, 32(4):361-367.

González G, Hinojo M, Mateo R, Medina A, Jiménez M: Occurrence of mycotoxin producing fungi in bee pollen. *International journal of food microbiology* 2005, 105(1):1-9.

Goulson D, Hughes WO: Mitigating the anthropogenic spread of bee parasites to protect wild pollinators. *Biological Conservation* 2015, 191:10-19.

Hajek AE, St Leger RJ: Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* 1994, 39:293-322.

Hammer Ø, Harper DAT, Ryan PD: PAST: Paleontological statistics software package for education and data analysis. *Paleontologica Electronica* 2001, 4.

Hefetz A, Errard C, Chambris A, LeNegrate A: Postpharyngeal gland secretion as a modifier of aggressive behavior in the myrmicine ant *Manica rubida*. *Journal of Insect Behavior* 1996, 9(5):709-717.

Herzner G, Strohm E: Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007, 17(2):R46-R47.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007, 33(4):849-859.

Herzner G, Engl T, Strohm E: Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011a, 82(2):321-328.

Herzner G, Ruther J, Goller S, Schulz S, Goettler W, Strohm E: Structure, chemical composition and putative function of the postpharyngeal gland of the emerald cockroach wasp, *Ampulex compressa* (Hymenoptera, Ampulicidae). *Zoology* 2011b, 114(1):36-45.

Herzner G, Schlecht A, Dollhofer V, Parzefall C, Harrar K, Kreuzer A, Pilsl L, Ruther J: Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials. *Proceedings of the National Academy of Sciences of the United States of America* 2013a, 110(4):1369-1374.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. *Plos One* 2013b, 8(12):e82780.

Hovelstad H, Leirset I, Oyaas K, Fiksdahl A: Screening analyses of pinosylvin stilbenes, resin acids and lignans in Norwegian conifers. *Molecules* 2006, 11(1):103-114.

Janzen DH: Why fruits rot, seeds mold, and meat spoils. *American Naturalist* 1977, 111(980):691-713.

Kainulainen P, Holopainen J: Concentrations of secondary compounds in Scots pine needles at different stages of decomposition. *Soil Biology and Biochemistry* 2002, 34(1):37-42.

Kaltenpoth M, Engl T: Defensive microbial symbionts in Hymenoptera. *Functional Ecology* 2014, 28(2):315-327.

Kaltenpoth M, Gottler W, Herzner G, Strohm E: Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 2005, 15(5):475-479.

Kaltenpoth M, Goettler W, Dale C, Stubblefield JW, Herzner G, Roeser-Mueller K, Strohm E: '*Candidatus* Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. *International Journal of Systematic and Evolutionary Microbiology* 2006, 56:1403-1411.

Kaltenpoth M, Schmitt T, Polidori C, Koedam D, Strohm E: Symbiotic streptomycetes in antennal glands of the South American digger wasp genus *Trachypus* (Hymenoptera, Crabronidae). *Physiological Entomology* 2010, 35(2):196-200.

Kaltenpoth M, Yildirim E, Guerbuez MF, Herzner G, Strohm E: Refining the roots of the beewolf-*Streptomyces* symbiosis: Antennal symbionts in the rare genus *Philanthinus* (Hymenoptera, Crabronidae). *Applied and Environmental Microbiology* 2012, 78(3):822-827.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(17):6359-6364.

Knecht D, Kaatz HH: Patterns of larval food production by hypopharyngeal glands in adult worker honey bees. *Apidologie* 1990, 21(5):457-468.

Koehler S, Doubsky J, Kaltenpoth M: Dynamics of symbiont-mediated antibiotic production reveal efficient long-term protection for beewolf offspring. *Frontiers in Zoology* 2013, 10.

Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, Strohm E, Svatos A: Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology* 2010, 6(4):261-263.

Lenoir A, Fresneau D, Errard C, Hefetz A: Individuality and colonial identity in ants: The emergence of the social representation concept. In: *Information processing in social insects.* Springer; 1999: 219-237.

Maddison WP, Maddison DR. Mesquite: A modular system for evolutionary analysis. Version 3.04. 2015. http://mesquiteproject.org.

Malinowski H, Sierpinska A: Occurrence and harmfulness of *Brachyderes incanus* L. (Coleoptera: Curculionidae) to young Scots pine (*Pinus sylvestris* L.) trees planted on post-fire areas. Insect Pathogens and Insect Parasitic Nematodes: *Melolontha*. *IOBC/wprs Bulletin* 2005, 28:169-173.

Maxfield-Taylor SA, Mujic AB, Rao S: First detection of the larval chalkbrood disease pathogen *Ascosphaera apis* (Ascomycota: Eurotiomycetes: Ascosphaerales) in adult bumble bees. *PloS one* 2015, 10(4):e0124868.

McArt SH, Koch H, Irwin RE, Adler LS: Arranging the bouquet of disease: Floral traits and the transmission of plant and animal pathogens. *Ecology Letters* 2014, 17(5):624-636.

Melo GAR: Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on the crabronid wasps. *Scientific Papers Natural History Museum the University of Kansas* 1999(14):1-55.

Noirot C, Quennedey A: Fine-structure of insect epidermal glands. *Annual Review of Entomology* 1974, 19:61-80.

O`Neill KM: Solitary wasps: Behavior and natural history: Cornell University Press; 2001.

Pagel M: Inferring the historical patterns of biological evolution. *Nature* 1999, 401(6756):877-884.

Phillips MA, Croteau RB: Resin-based defenses in conifers. *Trends in Plant Science* 1999, 4(5):184-190.

Polidori C, Boesi R, Isola F, Andrietti F: Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): The role of prey size. *European Journal of Entomology* 2005, 102(4):801-804.

Polidori C, Federici M, Papadia C, Andrietti F: Nest sharing and provisioning activity of females of the digger wasp, *Cerceiis rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 2006, 73(1):55-65.

Polidori C, Boesi R, Pesarini C, Papadia C, Bevacqua S, Federici M, Andrietti F: Temporal relationship between the prey spectrum and population structure of the weevil-hunting wasp *Cerceris arenaria* (Hymenoptera: Crabronidae). *Zoological studies* 2007, 46(1):83-91.

Ristich SS: The host relationship of a miltogrammid fly *Senotainia trilineata* (VDW). *Ohio Jour Sci* 1956, 56((5)):271-274.

Saviranta NM, Anttonen MJ, von Wright A, Karjalainen RO: Red clover (*Trifolium pratense* L.) isoflavones: determination of concentrations by plant stage, flower colour, plant part and cultivar. *Journal of the Science of Food and Agriculture* 2008, 88(1):125-132.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B *et al*: Fiji: An open-source platform for biological-image analysis. *Nature Methods* 2012, 9(7):676-682.

Schmid B, Schindelin J, Cardona A, Longair M, Heisenberg M: A high-level 3D visualization API for Java and ImageJ. *BMC Bioinformatics* 2010, 11.

Secil ES, Sevim A, Demirbag Z, Demir I: Isolation, characterization and virulence of bacteria from *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Biologia* 2012, 67(4):767-776.

Sikorowski PP, Lawrence AM, Inglis GD: Effects of *Serratia marcescens* on rearing of the tobacco budworm (Lepidoptera: Noctuidae). *American Entomologist* 2001, 47(1):51-60.

Soroker V, Vienne C, Hefetz A, Nowbahari E: The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 1994, 81(11):510-513.

Soroker V, Hefetz A, Cojocaru M, Billen J, Franke S, Francke W: Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 1995, 20(4):323-329.

Strohm E, Linsenmair KE: Leaving the cradle - How beewolves (*Philanthus triangulum* F) obtain the necessary spatial information for emergence. *Zoology-Analysis of Complex Systems* 1994, 98(3):137-146.

Strohm E, Linsenmair KE: Low resource availability causes extremely male-biased investment ratios in the European beewolf, *Philanthus triangulum* F (Hymenoptera, Sphecidae). *Proceedings of the Royal Society B-Biological Sciences* 1997, 264(1380):423-429.

Strohm E, Linsenmair KE: Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 2001, 26(2):198-203.

Strohm E, Marliani A: The cost of parental care: Prey hunting in a digger wasp. *Behavioral Ecology* 2002, 13(1):52-58.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36(2):113-122.

Strohm E, Herzner G, Kaltenpoth M, Boland W, Schreier P, Geiselhardt S, Peschke K, Schmitt T: The chemistry of the postpharyngeal gland of female European beewolves. *Journal of Chemical Ecology* 2008, 34(5):575-583.

Strohm E, Kaltenpoth M, Herzner G: Is the postpharyngeal gland of a solitary digger wasp homologous to ants? Evidence from chemistry and physiology. *Insectes Sociaux* 2010, 57(3):285-291.

Trienens M, Keller NP, Rohlfs M: Fruit, flies and filamentous fungi - Experimental analysis of animal-microbe competition using *Drosophila melanogaster* and *Aspergillus* mould as a model system. *Oikos* 2010, 119(11):1765-1775.

Van den Dool H, Kratz PD: A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography* 1963, 11:463-471.

Vander Wall SB: Food hoarding in animals: University of Chigaco Press; 1990.

Wagner GP. Homology, genes, and evolutionary innovation. 1st ed. Princeton: Princeton University Press; 2014.

Weiss K, Parzefall C, Herzner G: Multifaceted defense against antagonistic microbes in developing offspring of the parasitoid wasp *Ampulex compressa* (Hymenoptera, Ampulicidae). *PLoS ONE* 2014, 9(6).

Willers D, Lehmann-Danzinger H, Führer E: Antibacterial and antimycotic effect of a newly discovered secretion from larvae of an endoparasitic insect, *Pimpla turionellae*. *Archives of Microbiology* 1982, 133(3):225-229.

Zahradník J. Käfer Mittel-und Nordwesteuropas. 1st ed. Hamburg, Berlin: Parey; 1985.

# CHAPTER 3

# EVOLUTION AND PHYLOGENETIC DISTRIBUTION OF ANTIMICROBIAL BROOD DEFENSE IN BEEWOLVES (HYMENOPTERA, CRABRONIDAE): EVIDENCE FROM POSTPHARYNGEAL GLAND CHEMISTRY

Katharina Weiss, Erhard Strohm, Gudrun Herzner

## 3.1 Summary

In insects, long-chain hydrocarbons (HCs) serve important physiological and behavioral functions, including the prevention of dehydration and especially communication. One particularly intriguing function of HCs has evolved in the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae). Females embalm their paralyzed prey, honeybee workers (*Apis mellifera*), with a secretion from their large postpharyngeal gland (PPG). The high proportion of unsaturated HCs in the PPG secretion reduces the condensation of water on the prey, thus hampering fungal growth. This so-called prey embalming behavior has also been found in two closely related species, the Nearctic *Philanthus gibbosus* and the Neotropical *Trachypus elongatus*, both belonging to the same tribe, the Philanthini. Here, we investigate the chemical composition of PPG secretion in 27 species representing all three tribes and major phylogenetic clades of the Philanthinae to (1) assess whether prey embalming is a universal mechanism in the Philanthini, as has been hypothesized due to the occurrence of large and complex PPGs in all investigated species of this tribe, and (2) shed further light on the distribution of prey embalming in the other tribes of the Philanthinae. In female Philanthini, we found unsaturated hydrocarbons to be by far the predominant substance class, thus further sustaining the hypothesis that prey embalming as an antimicrobial brood protection mechanism is ubiquitous in this tribe. In the other two tribes, however, the PPG secretion of some species was dominated by methyl-branched hydrocarbons, but contains only relatively small proportions of unsaturated compounds, while in one species, *Aphilanthops frigidus*, the PPG contains approximately 50% unsaturated compounds, but also a considerable proportion of methyl-branched hydrocarbons. Therefore, the somewhat ambiguous results for Cercerini and Aphilanthopsini do not allow for general inferences about the use of PPG secretion in antimicrobial brood defense in these tribes.

## 3.2 Introduction

In insects, long-chain hydrocarbons (HCs) serve various physiological and behavioral functions. As part of the epicuticular lipid layer HCs are involved in the maintenance of water balance and the prevention of dehydration (Lockey 1980, Gibbs 1998, 2002). Yet cuticular HCs are also often both species and sex specific in social as well as solitary species (e.g. Singer 1998) and play a pivotal role in communication (e.g. Blomquist & Bagnères 2010). In social Hymenoptera in particular, cuticular HCs are essential in nestmate recognition and the maintenance of colony identity, as has been shown for honeybees, *Apis mellifera* (Dani et al. 2005), the social paper wasp *Polistes dominulus* (Dani et al. 2001), and European hornets, *Vespa crabo* (Ruther et al. 2002). In many ants HCs are constantly exchanged among nestmates generating the uniform colony odor (e.g. Crozier and Dix 1979, Hefetz et al. 1992, Soroker et al. 1994, 1995, 1998, Meskali et al. 1995, Vienne et al. 1995, Lenoir et al. 2001).

Yet, HCs can also function in antimicrobial defense. In the European beewolf *Philanthus triangulum* Fabricius (Hymenoptera, Crabronidae), females build subterranean brood cells in sandy soil and mass-provision their progeny with paralyzed honeybee workers (*A. mellifera*) (see e.g. Strohm & Linsenmair 1997, Strohm & Marliani 2002). Under the warm and humid conditions in the brood cells, the paralyzed honeybees and the larvae are exposed to a high risk of fungal infestation, which can severely reduce larval survival (Strohm 2000, Herzner et al. 2011a). Female *P. triangulum* effectively counter this threat by extensively licking the surface of the paralyzed prey (Strohm & Linsenmair 2001) and thereby applying large amounts of the lipid secretion to the prey's cuticle, a behavior that has been termed 'prey embalming' (Herzner & Strohm 2007, Herzner et al. 2007a). The source of the embalming secretion is a large head gland reservoir, the postpharyngeal gland (PPG) (Strohm et al. 2007), which has originally been described in ants where its primary function is the storage and mixing of the HCs involved in nestmate recognition (e.g. Hefetz et al. 1992, Soroker et al. 1994, 1995, 1998, Meskali et al. 1995, Vienne et al. 1995, Lenoir et al. 2001). In *P. triangulum*, the PPG secretion contains predominantly unsaturated HCs (Herzner et al. 2007a, Strohm et al. 2008) and consequently increases the proportion of unsaturated HCs on the prey's cuticle (Herzner & Strohm 2007, 2008). The resulting alteration of the physicochemical properties leads to a significant reduction of surficial water condensation and renders the microclimate on the prey`s surface unfavorable for fungal growth (Herzner & Strohm 2007). Accordingly, prey embalming retards the decomposition of the stored resources and increases larval survival (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2011a).

*Philanthus triangulum* belongs to the subfamily Philanthinae that comprises eight genera separated into three tribes (Alexander 1992): The two sister tribes Aphilanthopsini (comprising *Clypeadon* + *Aphilanthops*) and Cercerini (comprising (*Cerceris* + *Eucerceris*) + *Pseudoscolia*) and the Philanthini, the so-called beewolves (comprising *Philanthinus* + (*Philanthus* + *Trachypus*), the latter likely being a subgenus of *Philanthus* rather than a proper genus; Kaltenpoth et al. 2014). Generally, members of the Philanthinae provision their progeny with different species of aculeate Hymenoptera or Coleoptera, but show otherwise largely similar nesting behavior (e.g. Evans 1962, 1971, Bohart & Menke 1976, Evans & O'Neill 1988, Polidori et al. 2005, 2006). Therefore, all these species may largely face similar problems with regard to microbial infestation of the larval provisions (but see Weiss et al. 2015). Recently, we provided evidence that two species belonging to the same tribe as *P. triangulum* (Philanthini), the North America *Philanthus gibbosus* (Weiss et al. 2015) and the South American *Trachypus elongatus* (Herzner et al. 2013a), both also employ prey embalming with unsaturated HCs from their PPGs.

A comparative morphological study revealed that all female Philanthinae possess a PPG, albeit with marked morphological differences between the tribes. In all investigated species from both Aphilanthopsini and Cercerini the PPG is comprised of two pairs of simple tubes, while in the Philanthini females generally possess larger and more complex PPGs occupying a considerable part of the head capsule (Weiss et al. 2015). It may be assume that prey embalming probably requires a complex PPG with sufficient storage capacity and a large surface area for effective sequestration of HCs from the hemolymph (Strohm et al. 2008, 2010). Yet, the maintenance of such a gland is likely to entail costs (Herzner et al. 2011a). Therefore, based on their uniform PPG morphology, we hypothesized that species of the Philanthini generally deploy prey embalming to increase offspring survival, while this mechanism seems unlikely for Cercerini and Aphilanthopsini (Weiss et al. 2015).

In *P. triangulum*, the antifungal effect of prey embalming is mediated by unsaturated HCs that form a contiguous oily layer on the prey surface (Herzner & Strohm 2007) and the same most likely holds true for *P. gibbosus* and *T. elongatus* (Herzner et al. 2013a, Weiss et al. 2015). Further knowledge on the PPG chemistry of the Philanthinae may hence allow for inferences about the current distribution and evolutionary origin of prey embalming. Here we provide data on PPG chemistry of 24 species representing all major phylogenetic lineages of the Philanthini, as well as two species from the tribe Aphilanthopsini and one species from the tribe Cercerini. We, first, tested the prediction that the PPG of all Philanthini, supposedly all "prey embalmers", contains high proportions of unsaturated HCs and, second, try to clarify the picture for the Cercerini and

Aphilanthopsini, since the absence of high amounts of unsaturated HCs in their simple PPGs would further underpin the hypothesis that these tribes do not embalm their prey.

## 3.3 Methods

### 3.3.1 Specimens

Our comparative chemical investigation included one to six females each from 24 species of the tribe Philanthini (73 individuals in total, Table 3.1), representing the three major phylogenetic lineages of this tribe (Kaltenpoth et al. 2014): Five Palearctic/Afrotropical/Indian *Philanthus* species (including two subspecies of the European beewolf *P. triangulum*), 16 Nearctic *Philanthus* species, and three species of the Neotropical genus *Trachypus*. Moreover, one *Clypeadon* and one *Aphilanthops* species from North America (tribe Aphilanthopsini, six individuals in total), as well as one *Cerceris* species (tribe Cercerini, one individual) were included.

### 3.3.2 Chemical analysis

#### *3.3.2.1 Sample preparation*

For sampling of PPG content the wasps were cold anaesthetized, decapitated and their heads dissected under a stereomicroscope to remove the PPGs as described previously for *P. triangulum* (Herzner et al. 2007b). Alternatively, PPGs were removed by grasping the hypopharynx with tweezers and gently pulling the attached PPG out through the mouth (Strohm et al. 2007, 2008). Each gland was immediately immersed in hexane and remained there until chemical analysis was conducted. If dissections of heads for the removal of the PPG were not possible during field trips, entire heads of females were immersed in hexane for one hour.

**Table 3.1** Species included in the comparative chemical study of the postpharyngeal gland (PPG) content in female Philanthinae, with information on the phylogenetic affiliation of species (according to Kaltenpoth et al. 2014), the number of specimens examined (N), collection site of the species (Country), sample type (Sample; PPG: dissected PPGs, Head: whole heads), and which temperature program(s) was used for GC/MS analysis (GC; for details see text, section 3.3.2.2). The ID number assigned to each species (ID) is consistently used throughout the manuscript.

| Phylogenetic affiliation | ID | Species | N | Country | Sample | GC |
|---|---|---|---|---|---|---|
| Tribe Aphilanthopsini | 1 | *Aphilanthops frigidus* | 2 | USA | Head | 1 |
| | 2 | *Clypeadon laticinctus* | 4 | USA | Head | 1 |
| Tribe Cercerini | 3 | *Cerceris arenaria* | 1 | Germany | PPG | 1 |
| Palearctic/Afrotropical *Philanthus* | 4 | *Philanthus coronatus* | 1 | Germany | PPG | 1 |
| | 5 | *Philanthus loefflingi* | 3 | South Africa | Head | 1 |
| | 6 | *Philanthus rugosus* | 1 | South Africa | Head | 1 |
| | 7 | *Philanthus t. triangulum* | 5 | Germany | PPG/head | 1 /2 |
| | 8 | *Philanthus t. diadema* | 6 | South Africa | Head | 1 |
| Nearctic *Philanthus* | 9 | *Philanthus barbiger* | 2 | USA | Head | 1 |
| | 10 | *Philanthus basilaris* | 1 | USA | Head | 1 |
| | 11 | *Philanthus bicinctus* | 5 | USA | PPG/head | 1 |
| | 12 | *Philanthus crabroniformis* | 1 | USA | Head | 1 |
| | 13 | *Philanthus gibbosus* [1] | 4 | USA | Head | 1 |
| | 14 | *Philanthus gloriosus* | 5 | USA | PPG | 1 |
| | 15 | *Philanthus lepidus* | 2 | USA | Head | 1 |
| | 16 | *Philanthus multimaculatus* | 5 | USA | PPG | 1 |
| | 17 | *Philanthus pacificus* | 1 | USA | Head | 1 |
| | 18 | *Philanthus parkeri* | 2 | USA | Head | 1 |
| | 19 | *Philanthus psyche* | 6 | USA | Head | 1 |
| | 20 | *Philanthus pulcher* | 4 | USA | Head | 1 |
| | 21 | *Philanthus sanbornii* | 2 | USA | Head | 1 |
| | 22 | *Philanthus solivagus* | 2 | USA | Head | 1 |
| | 23 | *Philanthus ventilabris* | 2 | USA | Head | 1 |
| | 24 | *Philanthus zebratus* | 4 | USA | Head | 1 |
| *Trachypus* | 25 | *Trachypus boharti* [2] | 5 | Brazil | PPG | 3 |
| | 26 | *Trachypus elongatus* [2] | 3 | Brazil | PPG/head | 3 |
| | 27 | *Trachypus flavidus* | 1 | Brazil | Head | 1 |

[1] same GC samples as in Weiss et al. 2015, reanalyzed as described in 3.3.2.3
[2] same GC samples as in Herzner et al. 2013a, reanalyzed as described in 3.3.2.3

### *3.3.2.2 Capillary gas chromatography/mass spectrometry*

GC/MS analyses were performed with an Agilent 6890N Series gas chromatograph coupled to an Agilent 5973 inert mass selective detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a DB-5ms+ fused silica capillary column (length = 30 m, diameter = 250 µm; film thickness = 0.25 µm; Capital Analytical Ltd., Leeds, England). Generally, a split/splitless injector (250°C) was used in the splitless mode with the purge valve opened after 1 min; helium was used as carrier gas at a constant flow rate of 1 ml/min. The following temperature programs were used (Table 3.1):

(1) The GC was programmed from 60 to 300°C at a constant rate of 5°C/min, with an initial isothermal hold of 1 minute and a final isothermal hold of 10 minutes.
(2) The GC was programmed from 70 to 180°C at a rate of 30°C/min and then at 5°C/min to the final temperature of 310°C, with an initial isothermal hold of 1 minute and a final isothermal hold of 5 minutes.
(3) The GC was programmed from 70 to 180°C at 30°C/min and then at 3°C/min to 300°C, with an initial isothermal hold of 1 minute and a final isothermal hold of 5 minutes.

Electron ionization mass spectra were recorded at an ionization voltage of 70 eV, a source temperature of 230°C, and an interface temperature of 315°C. Data acquisition and storage were performed with the GC/MS software MSD ChemStation for Windows (Agilent Technologies, Palo Alto, CA, USA). Peak areas were obtained by manual integration using the GC/MS software.

*3.3.2.3 Substance identification*

The aim of the present study was a comparison of the proportion of substance classes among species, rather than the complete chemical characterization of the PPG secretion of single species. Most compounds were thus only identified to a certain degree that allowed for the assignment to a substance class (see section 3.3.3.1 below). *N*-Alkanes were identified by comparing their mass spectra and retention times with those of authentic reference compounds. For the other compounds, linear retention indices in relation to *n*-alkanes were calculated according to Van den Dool and Kratz (1963). Unsaturated HCs were tentatively identified by their characteristic mass spectra and linear retention indices. Double bond positions of alkenes and alkadienes were not determined, for alkatrienes double bond positions were tentatively determined by their characteristic fragmentation patterns and molecular ions (Witte et al. 2009). Tentative characterization of mono- and dimethyl alkanes was achieved by the diagnostic ions resulting from the typical cleavage at the branching positions, their molecular ion or a fragment at M-15 if the molecular ion could not be detected (Blomquist et al. 1987), as well as their linear retention indices. Saturated and mono-unsaturated ketones were tentatively identified as previously described (Herzner et al. 2013a). Double bond positions of unsaturated ketones were not determined.

### 3.3.3 Data analysis

#### *3.3.3.1 Data processing*

Different positional isomers of unsaturated HCs frequently co-eluted so that their peak areas had to be jointly integrated. The same was true for positional isomers of ketones, differing in the position of the carbonyl group. Each integrated peak (possibly representing more than one substance) was assigned to one of the following substance classes, separately for each chain length from C21 to C35: (1) Alkanes, (2) unsaturated HCs (including alkenes, alkadienes, and alkatrienes), (3) methyl-branched HCs (including mono- and dimethyl alkanes), and (4) ketones (including saturated and mono-unsaturated ketones). Some minor substances, presumably also HCs, could not be identified due to their incomplete mass spectra and were hence assigned to a separate "class" termed "unidentified". Since we found compounds with 15 different chain lengths, we could potentially have obtained a maximum of 61 categories [(4 classes x 15 chain lengths) + unidentified substances], yet 14 categories were not detected in any of the 80 analyzed individuals, leading to a final number of 47 categories in our dataset.

In one species, *Philanthus psyche*, trace amounts of heptacosen-4-one co-eluted with unsaturated C29s and had to be added to this category. Yet, the amount of heptacosen-4-one was vanishingly low as compared to the unsaturated C29s, hence, this procedure did not skew the proportions of substance categories in *P. psyche* as compared to other species. As heptacosen-4-one was the only ketone detected in this species, the proportion of ketones as a substance class could not be assessed in *P. psyche* (see Results).

For each individual, relative peak areas for each category (as %) were calculated by relating peak areas of the individual categories to the total peak area of all compounds in the sample. Some substance categories, like methyl-branched HCs with even chain lengths, were detected in only few individuals and generally in only very low proportions (>> 1%). Nonetheless, we did not exclude these categories from our dataset as this would have misleadingly increased the similarity between the samples.

#### *3.3.3.2 Statistical analysis: Cuticle extracts* vs. *dissected PPGs*

For *P. triangulum, T. elongatus*, and *Trachypus boharti* a high chemical congruency as well as a strong correlation between the relative amounts of components of head/whole body extracts and

of dissected PPGs has been demonstrated (Strohm et al. 2010, Herzner et al. 2013a). To further validate the use of extracts of dissected PPGs and head extracts in one comparative dataset, we tested for such a correlation in two more beewolf species, *Philanthus gloriosus* (N=5) and *Philanthus multimaculatus* (N=4), as well as in *Cerceris arenaria* (N=1).

Chemical data from whole body extracts of *P. gloriosus*, *P. multimaculatus*, and *C. arenaria* were obtained by GC/MS in the same way as described above for head/PPG extracts. The samples were analyzed with regard to both qualitative as well as quantitative (relative amounts of substance categories) congruency. Prior to the statistical analyses, the data were transformed to log-contrasts (Aitchison 1986, Kucera & Malmgren 1998). Since this transformation would make it necessary to exclude substance categories that do not occur in all samples (i.e. with an area of zero in any one sample), the original transformation was modified by adding a value of 0.001 to all relative areas, as well as the geometric means to avoid undefined values for substance categories. For *P. gloriosus* and *P. multimaculatus*, the means of the transformed values for each substance category were calculated. The relationship between the chemical profiles of surface and PPG extracts was analyzed by reduced major axis regressions on the mean proportions of the defined substance categories, for the three species separately. Quantitative chemical congruency between the samples can be assumed, if the slope of the regression line does not deviate significantly from 1 and the y-intercept does not deviate significantly from 0.

*3.3.3.3 Statistical analysis: Composition of PPG secretion*

To test whether basic PPG chemistry mirrors the patterns in PPG morphology in the Philanthinae, we conducted a non-metric multidimensional scaling analysis (nMDS) based on species means of arcsin (square root)-transformed values of all 47 defined substance categories (i.e. including both substance class and chain length information), using the Bray-Curtis-index as dissimilarity measure (Clark & Green 1988, Legendre & Legendre 1998). Species of the Cercerini and Aphilanthopsini were jointly assigned to one group, as they all possess comparably simple and similarly shaped PPGs (Weiss et al. 2015) and compared to the Philanthini, all characterized by more complex PPGs (Weiss et al. 2015). The Philanthini can be divided into a number of phylogenetic groups (Kaltenpoth et al. 2014). To test for a possible phylogenetic effect on the composition of substance categories, we also compared the three main clades within the Philanthini (i.e. Palearctic/Afrotropical/Indian *Philanthus*, Neotropical *Philanthus*, and *Trachypus*; Kaltenpoth et al. 2014). One-way analyses of similarity (ANOSIM), again based on Bray-Curtis-

indices, were used to test for significant differences between Cercerini/Aphilanthopsini and Philanthini, as well as between the three phylogenetic clades of the Philanthini.

All statistical analyses were performed with the statistics software package PAST (Version 2.08b) (Hammer et al. 2001). Values given are means ± standard deviation.

## 3.4 Results

Extracts of all 27 analyzed species and subspecies of the Philanthinae contained largely HCs with chain lengths ranging from C21 to C35, comprising linear alkanes, mono- and dimethyl-alkanes, and unsaturated linear HCs with one to three double bonds; moreover, a number of different long-chain mono-unsaturated and saturated ketones was detected (Table 3.2).

The comparison of the chemical profiles of dissected PPGs and cuticle washes of *P. gloriosus*, *P. multimaculatus*, and *C. arenaria* showed high chemical congruency for all three species. With one exception, all substance categories detected in PPG samples were also present in body extracts and *vice versa* in all three species (Figure 3.1). In *C. arenaria*, however, C22 was only detected in the PPG sample but not in the cuticle wash. In *P. gloriosus* and *P. multimaculatus*, the proportion of *n*-alkanes was somewhat higher in cuticular extracts than in PPG extracts, while the opposite was true for unsaturated HCs. In *C. arenaria*, however, *n*-alkanes seemed slightly overrepresented in PPG extracts. Yet, the relative amounts of substance categories showed a strong linear correlation between PPG and cuticle extracts that did not deviate significantly from direct proportionality [*P. gloriosus*: $p<0.001$, slope of the RMA regression line=1.031 (95% confidence intervals: 0.596 to 1.32), y-intercept=0.0 (95% confidence intervals: -0.708 to 0.836); *P. multimaculatus*: $p=0.003$, slope of the RMA regression line=1.491 (95% confidence intervals: 0.761 to 1.794), y-intercept=-0.006 (95% confidence intervals: -1.618 to 1.876); *C. arenaria*: $p<0.001$, slope of the RMA regression line=1.233 (95% confidence intervals: 0.402 to 1.632), y-intercept=0.0 (95% confidence intervals: -0.552 to 0.832)].

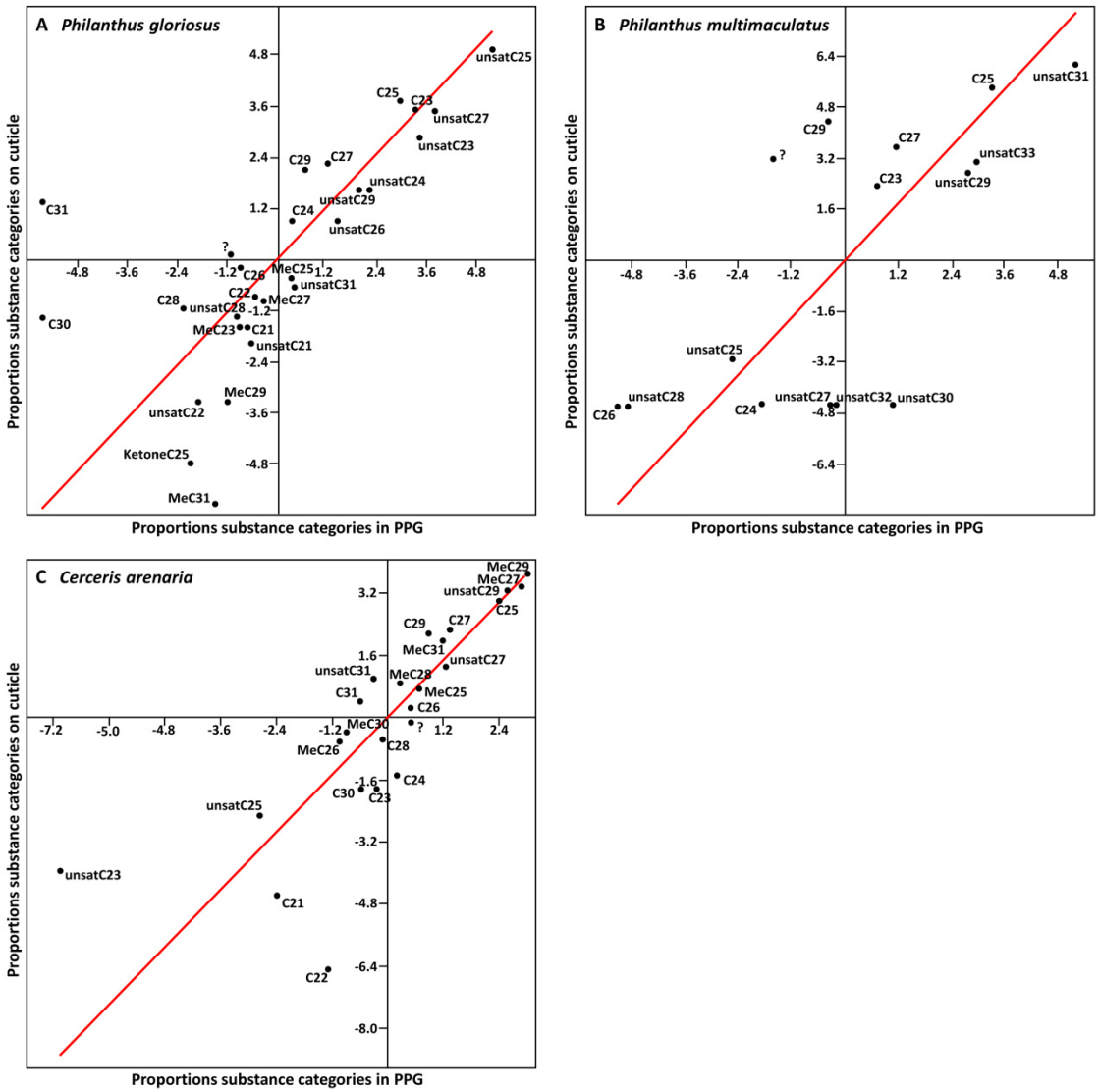


**Figure 3.1** Reduced major axis regressions for the proportions of substance categories (log-contrast-transformed values) in extracts of dissected PPGs and cuticular washes of whole bodies of females of (A) *Philanthus gloriosus* (N=5), (B) *Philanthus multimaculatus* (N=4), and (C) *Cerceris arenaria* (N=1). Color code represents substance classes: blue, n-Alkanes; green, unsaturated HCs; red, methyl-branched HCs; yellow, ketones; grey, unidentified substances. In all three species, there is a strong linear correlation in the proportions of substance categories in PPGs and on the body surface (all p-values < 0.01, see text for detailed statistics).

Whereas the proportions of alkanes varied only moderately among species (11.9% in *T. elongatus* to 32.9% in *Clypeadon laticinctus*), the proportions of unsaturated HCs and methyl-branched HCs showed vast interspecific variation (Figure 3.2). In *C. laticinctus* and *C. arenaria*, methyl-branched HCs (comprising mono- and also high amounts of dimethyl-alkanes) were the most abundant substance class. Notably, these two species showed the highest proportions of methyl-branched HCs (57.7±10.9% and 51.7%, respectively) and the lowest proportions of unsaturated HCs (3.1±1.6% and 19.8%) in this study. *Aphilanthops frigidus* had considerably lower relative amounts of methyl-branched HCs and higher amounts of unsaturated HCs than the former two species.

In all *Philanthus* and *Trachypus*, unsaturated HCs were the major substance class, accounting for 77.4±5.8% on average. Alkenes were the predominant unsaturated compounds, followed by alkadienes, and, in some species, small amounts of alkatrienes. In 14 of the 24 species of the Philanthini, we detected small amounts of long-chain ketones, ranging from C23 to C29 (Table 3.2). Generally, ketones made up only 0.5±1.1% on average of the extracted substances (Figure 3.2), with a maximum of 3.8±2.4% in *P. triangulum*. Methyl-branched HCs constituted only 0.6±0.6% on average of the total substance amount (Figure 3.2), with a maximum of 1.9±1.0% in *Philanthus sanbornii*

The nMDS analysis based on substance categories (including both composition of substance classes as well as chain length information) clearly separated *C. arenaria* and *C. laticinctus* (IDs 2 and 3) from all Philanthini, while *A. frigidus* (ID 1) lay between the other Cercerini/Aphilanthopsini and the Philanthini (Figure 3.3). A one-way ANOSIM yielded a significant difference in chemical composition between the Cercerini/Aphilanthopsini and the Philanthini (R=0.54, Bonferroni corrected p=0.006). The different phylogenetic clades within the Philanthini largely overlapped (Figure 3.3). A one-way ANOSIM comparing the different clades within the Philanthini showed no significant differences (over-all comparison: R=0.19, Bonferroni corrected p=0.065).

**Table 3.2** List of substance categories detected in in extracts of dissected PPGs and cuticular washes of heads of female Philanthinae (IDs correspond to Table 3.1). Note that, since only presence (x) and absence (empty cells) of substance categories based on the detection of their mass spectra is coded (regardless of whether they could be integrated separately for quantification), this table gives a more detailed listing of detected substance categories than was used for the statistical analysis (see section 3.3.3). Double bond positions of alkenes and alkadienes, as well as unsaturated ketones were not determined (see section 3.3.2.3).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Heneicosene | | | | x | | | x | | | | x | | | x | | | | | x | | x | | x | x | | | |
| *n*-Heneicosane | x | x | x | x | | | x | x | | | x | x | | x | x | | x | | x | | x | x | x | x | x | x | |
| Methylheneicosane | | | | | x | | | x | | | | | | | | | | | | | | | | | | | |
| Docosadiene | | | | | | | | x | | | | | | | | | | | | | | | | | | | |
| Docosene | | | | x | | | | x | | | x | | | x | | | | | | | | | | | | x | |
| *n*-Docosane | x | | x | x | x | x | x | x | | | x | | | x | x | | | | x | | | | x | x | x | x | x |
| Methyldocosane | | | | | | | | x | | | | | | | | | | | | | | | | | | | |
| Tricosadiene | x | | | x | | | x | x | | | x | x | | x | x | | | | | | x | | x | | | | |
| Tricosene | x | | | x | x | x | x | x | x | x | x | x | x | x | x | | x | | x | x | x | x | x | x | x | x | x |
| *n*-Tricosane | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Methyltricosane | x | x | | x | x | | x | x | | | x | | | x | x | | | | x | x | x | | x | | | x | |
| Tricosen-6-one | | | | | | | x | x | | | | | | | | | | | | | | | | | x | | |
| Tricosen-4-one | | | | | | | | x | | | | | | | | | | | | | | | | | x | | |
| Tricosan-6-one | | | | | | | | x | | | | | | | | | | | | | | | | | | | |
| Tricosan-4-one | | | | | | | x | x | | | | | | | | | | | | | | | | | x | | |
| Tetracosadiene | x | | | | | | | | | | x | | | x | | | | | | | | | | | | | |
| Tetracosene | x | | | x | x | x | x | x | | x | x | x | x | x | x | | | | | | x | x | x | x | x | x | x |
| *n*-Tetracosane | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Methyltetracosane | | | | | x | | | x | | | | | | | | | | | | | | | | | | | |
| Tetracosen-7-one | | | | | | | x | | | | | | | | | | | | | | | | | | | | |
| Pentacosatriene | | | | | | | | | | | | | | | x | | | | | | x | | | | | | |
| Pentacosadiene | x | | | x | x | x | x | x | x | x | x | x | | x | x | | x | | | | x | | x | x | x | | x |
| Pentacosene | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| *n*-Pentacosane | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Methylpentacosane | x | x | x | x | x | | x | x | | x | x | x | | x | x | x | | | x | | x | x | x | x | x | x | |

**Table 3.2** *continued*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dimethylpentacosane | x | | x | | | | | | | | x | | | x | | | | | | | | | | | | |
| Pentacosen-10-one | | | | | | | | | | | x | | | x | | | | | | | | | | | | | |
| Pentacosen-8-one | | | | | | | x | x | | x | x | x | | x | | | | | | | | | | | x | | |
| Pentacosen-6-one | | | | | | | x | x | | x | x | x | | x | | | | | | | | | | | x | | x |
| Pentacosen-4-one | | | | | | | | x | | | | | | | | | | | | | | | | | x | | |
| Pentacosan-8-one | | | | | | | x | x | | | | | | | | | | | | | | | | | | | |
| 3,6,9-Hexacosatriene | | | | | | | | | | | | | | | | | | | | | | | | | x | | |
| Hexacosadiene | | | | | | | | | | | x | | | | | | | | | | x | | | | | | |
| Hexacosene | | | | | x | x | x | x | x | x | x | x | x | x | x | x | x | | x | x | x | x | x | x | x | | x |
| *n*-Hexacosane | x | x | x | x | x | x | x | x | x | | x | x | | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Methylhexacosane | x | | x | | | | | | | | | | | | | | | | | | | | | | | | |
| Dimethylhexacosane | | | x | | | | | | | | | | | | | | | | | | | | | | | | |
| Hexacosen-9-one | | | | | | | x | x | | | | | | | | | | | | | | | | | | | |
| 3,6,9-Heptacosatriene | | | | | | | | | | | x | | | | x | | | | | | x | | | | x | | |
| Heptacosadiene | x | | | | | | x | x | x | x | x | | x | x | x | | x | | x | x | x | | x | x | x | | x |
| Heptacosene | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| *n*-Heptacosane | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Methylheptacosane | x | x | x | x | | | x | x | x | x | x | | x | x | x | | | | | x | x | x | x | x | x | x | x |
| Dimethylheptacosane | x | x | x | | | | | | | | x | | | x | | | | | | | | | | | | x | |
| Heptacosen-12-one | | | | | x | | | x | x | | | | | | | | | | | | | | | | | | |
| Heptacosen-10-one | | | | | x | | x | x | x | x | x | | | | | | | | | | x | | | x | x | | |
| Heptacosen-8-one | | | | | x | | x | | x | x | x | | | | | | | | | | x | | | x | x | | |
| Heptacosen-6-one | | | | | | | | | | | x | | | | | | | | | | | | | | | | |
| Heptacosen-4-one | | | | | | | | x | | x | | | x | | | | | | x[1] | | | | | x | | | |
| Heptacosan-12-one | | | | | | | x | | | | | | | | | | | | | | | | | | | | |
| Heptacosan-10-one | | | | | | | x | x | | | | | | | | | | | | | | | | | | | |
| 3,6,9-Octacosatriene | | | | | | | | | | | | | | | | | | | | | x | | | | | | |
| Octacosadiene | | | | | | | | | | | | | | | | | | x | | x | x | | | | | | |
| Octacosene | | | | | x | | x | x | x | x | x | | x | x | | x | x | x | x | x | x | x | x | x | x | x | |
| *n*-Octacosane | | | x | | x | | x | x | x | | x | | | x | | | | | x | x | x | | x | x | | x | |
| 3,6,9-Nonacosatriene | | | | | | | | | | | x | | | | | | | | x | | x | | | | x | | |
| Nonacosadiene | x | | | | | | | | x | x | x | | | x | | x | | | x | x | x | | x | x | x | x | |
| Nonacosene | x | | x | | x | x | x | x | x | x | x | x | x | x | x | x | x | | x | x | x | x | x | x | x | x | x |
| *n*-Nonacosane | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | | x | x | x | x | x | x | x | x | x |
| Methylnonacosane | x | x | x | | | | x | x | x | x | x | | | x | x | | | | | x | x | | x | x | | x | |
| Dimethylnonacosane | x | x | x | | | | | | | | x | | | | | | | | | | | | | | | | |
| Nonacosen-12-one | | | | | x | | | | | x | | | | | | | | | | | x | | | | x | | |
| Nonacosen-10-one | | | | | | | | | | | | | | | | | | | | | x | | | | x | | |
| Nonacosen-6-one | | | | | x | | | | | | | | x | | | | | | | | | | | | | | |
| Nonacosan-10-one | | | | | | | | x | | | | | | | | | | | | | | | | | | | |
| Triacontadiene | | | | | | | | | | | | | | | | | | | | x | | | | | | | |
| Triacontene | x | | | | x | x | | | x | x | x | | | | | x | | | x | x | x | | x | | x | x | |
| *n*-Triacontane | | | x | | x | x | x | x | | | x | | | | | | | | | | x | | | x | | x | |
| 3,6,9-Hentriacontatriene | | | | | | | | | | | | | | | | | | | x | | x | | | | | | |
| Hentriacontadiene | x | | | | | | | | x | x | x | | | | x | x | | x | | x | x | | | | x | x | |
| Hentriacontene | x | | x | | x | x | x | x | x | x | x | | x | x | x | x | | x | x | x | x | x | x | x | x | x | |
| *n*-Hentriacontane | x | x | x | | x | x | x | x | | | x | | | | x | x | | | | | x | | x | x | | x | |
| Methylhentriacontane | x | x | x | | x | | x | x | | | x | | | x | | | | | | | x | | x | x | | x | |
| Dimethylhentriacontane | x | x | | | | | | | | | x | | | | | | | | | | | | | | | | |
| Dotriacontadiene | | | | | | | | | | | | | | | | x | | | | x | | | | | | x | |
| Dotriacontene | | | | | x | x | | | | | | | | | | x | | | | | x | | | | | x | |
| *n*-Dotriacontane | | | | | | | | | | | | | | | | | | | | | | | | | | x | |
| Tritriacontadiene | | | | | | | | | | | | | | | | | | x | | x | | | | | | x | |
| Tritriacontene | x | | | | | | x | | | | | | | | | x | | x | | | x | | x | | | x | |
| *n*-Tritriacontane | x | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Methyltritriacontane | x | x | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dimethyltritriacontane | x | x | | | | | | | | | | | | | | | | | | | | | | | | | |
| Tetratriacontene | | | | | | | | | | | | | | | | | | | | | | | | | | x | |
| Pentatriacontadiene | | | | | | | | | | | | | | | | | | | | | x | | | | | x | |
| Pentatriacontene | | | | | | | | | | | | | | | | | | | | | x | | | | | x | |
| Methylpentatriacontane | | | | | | | | | | | | | | | | | | | | | x | | | | | | |
| Unidentified substances | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | | x | x | x | x | x | x | x | x | x |

[1] In *Philanthus psyche* (ID 19) trace amounts of heptacosen-4-one were detected, but co-eluted with unsaturated C29s; thus, for *P. psyche*, ketones could not be included in the comparison of the proportion of substance classes (see section 3.3.3.1 and Figure 3.1 for details).

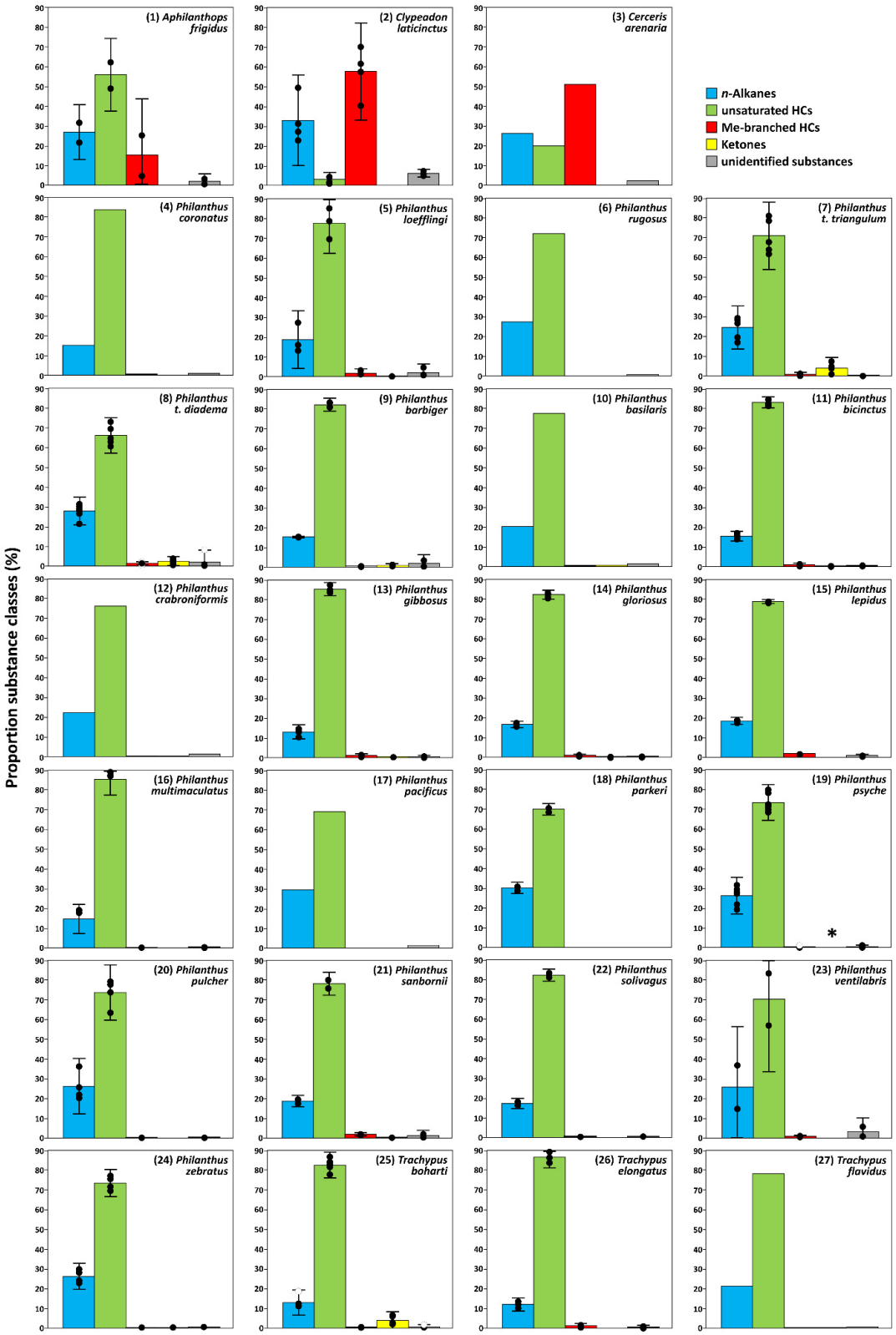


**Figure 3.2** Mean proportions (bars) ± standard deviation (whiskers) of *n*-alkanes, unsaturated hydrocarbons, methyl-branched hydrocarbons, ketones, and unidentified substances in extracts of female Philanthinae (numbers in parentheses are species IDs corresponding to Table 3.1). Color code for substance classes see legend in figure. Filled circles show individual data points for species for which more than three individuals had been investigated, open circles indicate outliers (included in statistical analysis).

***** Note that for *Philanthus psyche* (ID 19) trace amounts of heptacosen-4-one were detected that co-eluted with unsaturated C29s; thus, the proportion of ketones could not be assessed in this species (see text, section 3.3.3.1).

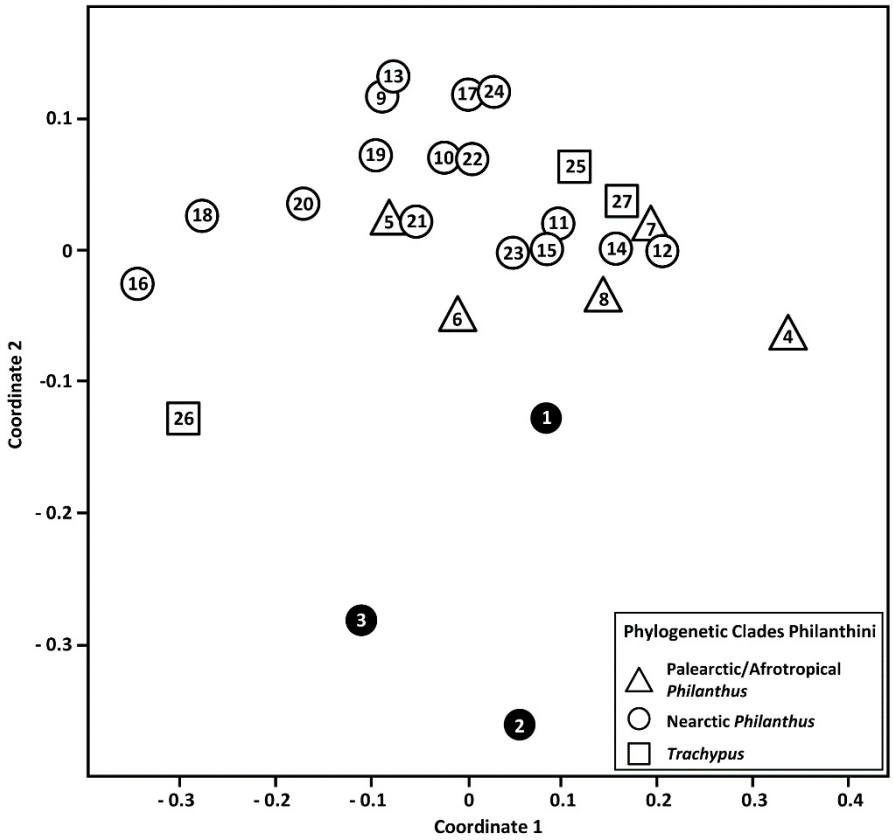


**Figure 3.3** Two-dimensional non-metric MDS representation of the chemical profiles (based on substance categories) of individual female Philanthinae (stress value=0.113). Numbers are species IDs corresponding to Table 3.1. Species of the Aphilanthopsini (IDs 1 and 2)/Cercerini (ID 3) (filled symbols) differ significantly from the Philanthini (open symbols; for the coding of phylogenetic clades see legend in figure) (one-way ANOSIM, R = 0.54, Bonferroni corrected p = 0.006).

## 3.5 Discussion

All PPG/cuticle extracts of female Philanthinae contained principally HCs, with rather little interspecific variation in the proportion of alkanes, but large differences in the proportion of unsaturated HCs and methyl-branched HCs. In *P. multimaculatus*, *P. gloriosus*, as well as *C. arenaria* we show that the chemical profiles of the PPG and the cuticle are highly congruent. This is in line with findings in ants (Bagnères & Morgan 1991, Lucas et al. 2004, Akino et al. 2004), the cockroach wasp *Ampulex compressa* (Herzner et al. 2011b), as well as the previously investigated Philanthini *P. triangulum* (Strohm et al. 2010), *T. boharti*, and *T. elongatus* (Herzner et al. 2013a) and for the first time expands this general finding to a member of the tribe Cercerini for which the chemical composition of the PPG secretion has not previously been investigated. In fact, the chemical resemblance of cuticular washes of heads (as used in our dataset) and dissected PPGs is likely even closer as compared to whole body extracts. For both ants and *P. triangulum* it has been shown or proposed that this congruence comes about as the HCs are synthesized by

oenocytes in the fat body and subsequently distributed to both the cuticle and the PPG via the hemolymph (Soroker & Hefetz 2000, Lucas et al. 2004, Strohm et al. 2010). The common chemical match of PPG and cuticle justifies the inclusion of head extracts in our chemical and statistical analyses and enabled us to evaluate our predictions based on a much larger data set.

In the Philanthini unsaturated HCs account for the lion's share of compounds, while methyl-branched HCs are present in only very small proportions. *A. frigidus* (Aphilanthopsini) still comprises more than 50% unsaturated HCs, but also considerably higher proportions of methyl-branched alkanes than the Philanthini. In *Clypeadon laticinctus* (Aphilanthopsini) and *C. arenaria* (Cercerini) methyl-branched HCs constitute the highest proportions of the HCs, while unsaturated hydrocarbons are present in comparably small relative amounts.

Multivariate statistical analyses based on substance categories revealed that the HC profiles of the Philanthini differed significantly from those of the Cercerini and Aphilanthopsini. This finding mirrors the pattern of PPG morphology among the Philanthinae, where the basal Cercerini/Aphilanthopsini possess rather small and simple PPGs that are distinct from the large and complex-shaped PPGs of the more derived Philanthini (Weiss et al. 2015). A previously performed ancestral state reconstruction suggests that the tube-shaped PPGs of the Cercerini/Aphilanthopsini are the ancestral gland "type" within the Philanthinae. The large and mostly glove-shaped PPGs seem to have evolved after the branching off of the Philanthini and represent an autapomorphy of this tribe (Weiss et al. 2015).

Notably, there was virtually no variation in the composition of substance classes found in the PPG/head extracts among the 24 investigated species of the Philanthini and no trends among phylogenetic clades despite the fact that the comparison comprised samples collected over the course of several years and analyzed with different GC/MS setups, and was based on rather small numbers of specimens per species. The low variation in PPG chemistry reflects the rather uniform morphology of this gland in the Philanthini (Weiss et al. 2015).

The species that have so far been shown to embalm their prey, *P. triangulum*, *P. gibbosus*, and *T. elongatus*, belong to the Philanthini and, thus, bear large, complex PPGs (Herzner et al. 2007a, 2013a, Strohm et al. 2007, Weiss et al. 2015) and the PPG secretions of these "prey embalmers" are characterized by high proportions of unsaturated HCs (Herzner et al. 2007a, 2013a, Strohm et al. 2008, Weiss et al. 2015). Based on the finding that all Philanthini investigated to date possess large and complex PPGs and the assumption that they all face similar threats with regard to fungal

infestations of their larval provisions, we hypothesized that the Philanthini generally embalm their prey with PPG secretion to counter the infestation by mold fungi (Weiss et al. 2015). The antifungal effect of prey embalming has been shown to depend on a high degree of unsaturation of the secretion that is used to coat the prey (Herzner & Strohm 2007). Therefore, we had further predicted that their PPGs contain predominantly unsaturated HCs. Our findings that the proportion of unsaturated HCs is generally extraordinarily high in all investigated Philanthini, therefore, provide further evidence in favor of this hypothesis.

This is all the more true, as the predominance of unsaturated HCs in the PPG is by no means universal. Rather, the different Hymenopteran species (mostly ants) for which data is available, exhibit considerable variation in HC composition of PPG content, ranging from high proportions of unsaturated HCs in the two Formicinae *Formica selysi* (Bagnères & Morgan 1991) and *Formica japonica* (Akino et al. 2004) and the predominance of alkanes in *Manica rubida* (Myrmicinae) (Bagnères & Morgan 1991), to various formicine and myrmicine species possessing predominantly, or even almost exclusively, methyl-branched HCs in their PPGs (Bagnères & Morgan 1991, Soroker & Hefetz 2000, Lucas et al. 2004). As demonstrated for *Cataglyphis*, there can even be considerable variation in PPG chemistry within one genus (Dahbi et al. 1996).

We are aware that high proportions of unsaturated HCs in the PPG alone do not provide sufficient evidence for prey embalming in the respective species. Female cockroach wasps, *A. compressa*, for example, likewise containing primarily unsaturated HCs in their PPG, do not embalm their prey (Herzner et al. 2011b). However, *A. compressa* has a very small PPG compared to beewolves (Herzner et al. 2011b).

Yet, the joint occurrence of large PPGs and the marked predominance of unsaturated HCs in PPG secretions, may serve as a reliable indicator for prey embalming. In *P. triangulum* (Herzner & Strohm 2007, Herzner et al. 2007a), prey embalming significantly increases both the total amount of cuticular HCs and the proportion of unsaturated components, while in both *P. gibbosus* and *T. elongatus* only the proportion of unsaturated substances, but not the total amount of HCs, increases significantly (Herzner et al. 2013a, Weiss et al. 2015). Yet, in all species, females still have to apply considerable amounts of unsaturated HCs to increase the proportion of these compounds on the preys' surface for the physicochemical mechanism of prey preservation to take effect (Herzner & Strohm 2007, Strohm et al. 2008, Herzner et al. 2011a). Taken together, the hypothesis that prey embalming is widespread in the Philanthini is supported by several observations: (1) their large and complex PPGs, (2) the high amounts of unsaturated HCs in their

PPGs, (3) the fact that all so far investigated Philanthini apply HCs from their PPG to their prey, and that (4) the three previously described "prey embalmers" (the Palearctic/Afrotropical *P. triangulum*, the Nearctic *P. gibbosus*, and the Neotropical *T. elongatus*) represent the major phylogenetic clades of the Philanthini.

On the other hand, the rather small PPGs of *C. laticinctus* and *C. arenaria* that contain very low proportions of unsaturated HCs, make prey embalming in these species unlikely. Indeed, there is evidence that the surface chemistry of provisioned beetles taken from nests of *C. arenaria* does not differ from control beetles (Wurdack et al., *personal communication*). As for *A. frigidus*, whose PPG contains considerably higher amounts of methyl-branched HCs than in the Philanthini, but also about 50% of unsaturated HCs, the situation is less clear. Interestingly, the PPG of *A. frigidus*, though still smaller and far less complex than in the Philanthini, is yet somewhat more voluminous then the PPGs of *C. laticinctus* and all investigated Cercerini (Weiss et al. 2015).

The occurrence of long-chain saturated and unsaturated ketones in more than half of the investigated Philanthini is noteworthy. Though ketones of this kind have previously been described from *P. triangulum*, *P. gibbosus*, and *T. elongatus* (Strohm et al. 2008, Herzner et al. 2013a, Weiss et al. 2015), they seem to be uncommon in insects at large. The rare incidences include the two saturated ketones heptacosan-10-one and heptacosan-12-one, as well as (*Z*)-18-heptacosen-10-one that form part of the contact sex pheromone of female white-spotted longicorn beetles, *Anoplophora malasiaca* (Yasui et al. 2003), and an heptacosen-12-one that has been detected on the cuticle of the house fly *Musca domestica* (Mpuru et al. 2001). Notably, in *P. triangulum*, 16-pentacosen-8-one and 18-heptacosen-10-one are also comprised in the PPG secretion of males, which is used to mark territories for mate attraction (Kroiss et al. 2006).

The function of the long-chain ketones in the PPG secretion remains to be explained. They could provide some direct antimicrobial effect in addition to the physicochemical effect of unsaturated HCs, but a strong effect seems unlikely, however, since in the European beewolf *P. triangulum* the PPG secretion (that contains nearly 4% ketones) had no direct antimicrobial effect in inhibition zone assays (Herzner et al. 2007a).

For *P. triangulum* it has been shown that prey embalming enhances offspring survival (Herzner et al. 2011a), which has significant positive effects on the females' reproductive success, and it stands to reason that the other beewolves benefit from this kind of prey preservation just as much. But why then do not all Philanthinae embalm their prey? One possible explanation is that

the maintenance of a complex PPG and the production of large amounts of HCs also entail costs for the females (Herzner et al. 2011a). For prey embalming to evolve and persist, its benefits must outweigh these costs.

The honeybee prey of *P. triangulum* is highly susceptible to mold fungi when stored under the warm and humid conditions that usually prevail in brood cells (Herzner & Strohm 2007, Herzner et al. 2011a). The other Philanthini, which nest under comparable conditions and mostly rely on different bee species, or occasionally other aculeate wasps, as larval provisions (e.g. Evans & O`Neill 1988), most probably face similar threats. Bees and wasps are supposedly more susceptible to fungal infestations in the nest than the ant prey of the Aphilanthopsini (Ristich 1956, Evans 1962) and the coleopteran prey (mainly weevils and buprestids, e.g. Bohart & Menke 1976) of most Cercerini (including the here investigated *C. arenaria*) (for details see Weiss et al. 2015 and references therein). The Philanthini may hence have experienced stronger selection pressures regarding prey preservation than most species of the Cercerini and Aphilanthopsini.

Further evidence that the Philanthini experience exceptional threats by detrimental microbes comes from another antimicrobial strategy of beewolves, the symbiosis with antibiotic-producing *Streptomyces* bacteria (Kaltenpoth et al. 2006) that protect the beewolf cocoons from a broad range of microbes during hibernation (Kaltenpoth et al. 2005, Kroiss et al. 2010, Koehler et al. 2013). The symbiotic bacteria have been found in *Philanthus* (Kaltenpoth et al. 2006, 2014), as well as the two other genera of the Philanthini, *Trachypus* (Kaltenpoth et al. 2010, 2014) and *Philanthinus* (Kaltenpoth et al. 2012, 2014). Yet, all Cercerini and Aphilanthopsini investigated thus far (namely members of the genera *Cerceris*, *Aphilanthops*, and *Clypeadon*) do not engage in this protective symbiosis (Kaltenpoth et al. 2006).

Whether bees are in fact a 'riskier' prey with respect to fungal infestations and have necessitated the evolution of prey embalming as an effective countermeasure, could possibly be elucidated by further investigating prey preservation in the genus *Cerceris*. The majority of the about 850 *Cerceris* species prey upon Coleoptera, but some have adapted to bee-hunting (e.g. Bohart & Menke 1976). Given that the use of bees as larval food source was indeed a driving factor for the evolution of prey embalming in the Philanthini, it might be hypothesized that bee-hunting *Cerceris* possess some kind of prey preservation strategy. Indeed, while the beetle-hunting *C. arenaria* almost certainly does not embalm its prey (see above), Wurdack et al. (*personal communication*) found that provisioned bees taken from the nests of bee-hunting *Cerceris* possess higher amounts of substances on their surface that are characteristic for the surface chemistry of the respective

*Cerceris* females as compared to control bees without contact to the wasps and, thus, hypothesized that bee-hunting *Cerceris* species do employ some kind of prey embalming.

In this regard, further studies are also needed for the Aphilanthopsini. The two yet investigated species from this tribe, *C. laticinctus* and *A. frigidus*, both hunt for ants, yet while *C. laticinctus* resembles the probably non-embalming *C. arenaria* in both PPG morphology (Weiss et al. 2015) and chemistry, *A. frigidus* seems to constitute an intermediate stage between *C. arenaria*/*C. laticinctus* and the Philanthini (see above).

The deployment of antimicrobial secretions has been documented for a diverse range of insects, including solitary wasps (Willers et al. 1982, Führer & Willers 1986, Herzner et al. 2013b, Weiss et al. 2014) and bees (Cane et al. 1983), ants (Brough 1983, Bot et al. 2002, Rodrigues et al. 2008, de Lima Mendoça et al. 2009, Fernández-Marín et al. 2015), dipteran maggots (e.g. Jaklič et al. 2008, Pöppel et al. 2014), different beetles (Gross et al. 1998, 2002, 2008, Prendeville & Stevens 2002, Arce et al. 2012, 2013, Pedrini et al. 2015), and termites (Bulmer et al. 2009, Hamilton et al. 2011). In all these taxa the secretions have direct antimicrobial effects. Prey embalming in beewolves, however, uniquely builds on a simple yet effective indirect physicochemical effect. The increase of unsaturated compounds in the prey's surface HCs creates an oily coating that reduces surficial water condensation and deprives molds of the water they need to germinate (Herzner & Strohm 2007). This pivotal and unique role of unsaturated HCs in beewolves can explain the consistent evolution of female PPG chemistry across all phylogenetic lineages of the Philanthini.

## 3.6 References


Aitchison J: The statistical analysis of compositional data. Series: Monographs on Statistics and Applied Probabilities. London: Chapman & Hall; 1986.

Akino T, Yamamura K, Wakamura S, Yamaoka R: Direct behavioral evidence for hydrocarbons as nestmate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Applied Entomology and Zoology* 2004, 39(3):381-387.

Alexander BA. A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 1992, 17(2):91-108.

Arce A, Johnston P, Smiseth P, Rozen D: Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology* 2012, 25(5):930-937.

Arce AN, Smiseth PT, Rozen DE: Antimicrobial secretions and social immunity in larval burying beetles, *Nicrophorus vespilloides*. *Animal Behaviour* 2013, 86(4):741-745.

Bagnères AG, Morgan ED: The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 1991, 47(1):106-111.

Blomquist GJ, Bagnères AG: Insect hydrocarbons: biology, biochemistry, and chemical ecology: Cambridge University Press; 2010.

Blomquist GJ, Nelson DR, Derenobales M: Chemistry, biochemistry, and physiology of insect cuticular lipids. *Archives of Insect Biochemistry and Physiology* 1987, 6(4):227-265.

Bohart RM, Menke AS. Sphecid wasps of the world: a generic revision. 1st ed. Ithaca: University of California Press; 1976.

Bot ANM, Ortius-Lechner D, Finster K, Maile R, Boomsma JJ: Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux* 2002, 49(4):363-370.

Brough EJ: The antimicrobial activity of the mandibular gland secretion of a formicine ant, *Calomyrmex sp.* (Hymenoptera, Formicindae). *Journal of Invertebrate Pathology* 1983, 42(3):306-311.

Bulmer MS, Bachelet I, Raman R, Rosengaus RB, Sasisekharan R: Targeting an antimicrobial effector ction in insect immunity as a pest control strategy. *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106:12652–12657.

Cane JH, Gerdin S, Wife G: Mandibular gland secretions of solitary bees (Hymenoptera, Apoidea) - potential for nest cell disinfection. *Journal of the Kansas Entomological Society* 1983, 56(2):199-204.

Clarke K, Green R: Statistical design and analysis for a 'biological effects' study. *Marine Ecology Progress Series* 1988, 46:213-226.

Crozier RH, Dix MW: Analysis of 2 genetic models for the innate components of colony odor in social Hymenoptera. *Behavioral Ecology and Sociobiology* 1979, 4:217-224

Dahbi A, Lenoir A, Tinaut A, Taghizadeh T, Francke W, Hefetz A: Chemistry of the postpharyngeal gland secretion and its implication for the phylogeny of Iberian *Cataglyphis* species (Hymenoptera: Formicidae). *Chemoecology* 1996, 7(4):163-171.

Dani FR, Jones GR, Destri S, Spencer SH, Turillazzi S: Deciphering the recognition signature within the cuticular chemical profile of paper wasps. Animal Behaviour 2001, 62:165–171.

Dani FR, Jones GR, Corsi S. Beard R, Pradella D, Turillazzi S.: Nestmate recognition cues in the honey bee: differential importance of cuticular alkanes and alkenes. Chemical Senses 2005, 30:477–489.

de Lima Mendonça AD, da Silva CE, de Mesquita FLT, da Silva Campos R, Do Nascimento RR, Ximenes ECPA, Sant'Ana AEG: Antimicrobial activities of components of the glandular secretions of leaf cutting ants of the genus *Atta*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2009, 95(4):295-303.

Evans HE: A review of nesting behavior of digger wasps of the genus *Aphilanthops*, with special attention to the mechanism of prey carriage. *Behaviour* 1962, 19:239-260.

Evans HE: Observations on the nesting behavior of wasps of the tribe Cercerini. *Journal of the Kansas Entomological Society* 1971, 44:500-523.

Evans HE, O' Neill KM: The natural history and behavior of North American beewolves. 1st ed. Ithaca: Cornell University Press; 1988.

Fernández-Marín H, Nash DR, Higginbotham S, Estrada C, van Zweden JS, d'Ettorre P, Wcislo WT, Boomsma JJ: Functional role of phenylacetic acid from metapleural gland secretions in controlling fungal pathogens in evolutionarily derived leaf-cutting ants. *Proceedings of the Royal Society B-Biological Sciences* 2015, 282(1807).

Führer E, Willers D: The anal secretions of the endoparasitic larva *Pimpla turionellae*: Sites of production and effects *Journal of Insect Physiology* 1986, 2:361-367.

Gibbs AG: Water-proofing properties of cuticular lipids. *American Zoologist* 1998, 471-482.

Gibbs AG: Lipid melting and cuticular permeability: new insights into an old problem. *Journal of Insect Physiology* 2002, 48(4): 391-400.

Gross J, Müller C, Vilcinskas A, Hilker M: Antimicrobial activity of exocrine glandular secretions, hemolymph, and larval regurgitate of the mustard leaf beetle *Phaedon cochleariae*. *Journal of Invertebrate Pathology* 1998, 72(3):296-303.

Gross J, Podsiadlowski L, Hilker M: Antimicrobial activity of exocrine glandular secretion of *Chrysomela* larvae. *Journal of Chemical Ecology* 2002, 28(2):317-331.

Gross J, Schumacher K, Schmidtberg H, Vilcinskas A: Protected by fumigants: Beetle perfumes in antimicrobial defense. *Journal of Chemical Ecology* 2008, 34(2):179-188.

Hamilton C, Lay F, Bulmer MS: Subterranean termite prophylactic secretions and external antifungal defenses. *Journal of Insect Physiology* 2011, 57:1259–1266.

Hammer Ø, Harper DAT, Ryan PD: PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 2001, 4.

Hefetz A, Errard C, Cojocaru M: Heterospecific Substances in the Postpharyngeal Gland Secretion of Ants Reared in Mixed Groups. *Naturwissenschaften* 1992, 79(9):417-420.

Herzner G, Strohm E. Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007;17:R46–7.

Herzner G, Strohm E: Food wrapping by females of the European Beewolf, *Philanthus triangulum*, retards water loss of larval provisions. *Physiological Entomology* 2008, 33(2):101-109.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007a, 33:849–59.

Herzner G, Goettler W, Kroiss J, Purea A, Webb AG, Jakob PM, Roessler W, Strohm E: Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 2007b, 36(2):123-133.

Herzner G, Engl T, Strohm E. Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011a, 82:321–8.

Herzner G, Ruther J, Goller S, Schulz S, Goettler W, Strohm E. Structure, chemical composition and putative function of the postpharyngeal gland of the emerald cockroach wasp, *Ampulex compressa* (Hymenoptera, Ampulicidae). *Zoology* 2011b, 114:36–45.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. *PLoS One* 2013a, 8.

Herzner G, Schlecht A, Dollhofer V, Parzefall C, Harrar K, Kreuzer A, Pilsl L, Ruther J: Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials. *Proceedings of the National Academy of Sciences of the United States of America* 2013b, 110:1369–74.

Jaklič D, Lapanje A, Zupančič K, Smrke D, Gunde-Cimerman N: Selective antimicrobial activity of maggots against pathogenic bacteria. *Journal of medical microbiology* 2008, 57(5):617-625.

Kaltenpoth M, Gottler W, Herzner G, Strohm E. Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 2005, 15:475-479.

Kaltenpoth M, Goettler W, Dale C, Stubblefield JW, Herzner G, Roeser-Mueller K, Strohm E: '*Candidatus* Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. *International Journal of Systematic and Evolutionary Microbiology* 2006, 56:1403-1411.

Kaltenpoth M, Schmitt T, Polidori C, Koedam D, Strohm E: Symbiotic streptomycetes in antennal glands of the South American digger wasp genus *Trachypus* (Hymenoptera, Crabronidae). *Physiological Entomology* 2010, 35(2):196-200.

Kaltenpoth M, Yildirim E, Guerbuez MF, Herzner G, Strohm E: Refining the roots of the beewolf-*Streptomyces* symbiosis: antennal symbionts in the rare genus *Philanthinus* (Hymenoptera, Crabronidae). *Applied and Environmental Microbiology* 2012, 78(3):822-827.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(17):6359-6364.

Koehler S, Doubsky J, Kaltenpoth M: Dynamics of symbiont-mediated antibiotic production reveal efficient long-term protection for beewolf offspring. *Frontiers in Zoology* 2013, 10.

Kroiss J, Schmitt T, Schreier P, Strohm E, Herzner G: A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 2006, 32(12):2763-2776.

Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, Strohm E, Svatos A: Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology* 2010, 6(4):261-263.

Kucera M, Malmgren BA: Logratio transformation of compositional data: a resolution of the constant sum constraint. *Marine Micropaleontology* 1998, 34(1):117-120.

Legendre P, Legendre L: Numerical Ecology. Amsterdam: Elsevier Science B.V; 1998.

Lenoir A, Hefetz A, Simon T, Soroker V: Comparative dynamics of gestalt odour formation in two ant species *Camponotus fellah* and *Aphaenogaster senilis* (Hymenoptera : Formicidae). *Physiological Entomology* 2001, 26(3):275-283.

Lockey KH: Insect cuticular hydrocarbons. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 1980, 65(3):457-462.

Lucas C, Pho D, Fresneau D, Jallon J: Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*. *Journal of Insect Physiology* 2004, 50(7):595-607.

Meskali M, Bonavita-Cougourdan A, Provost E, Bagnères AG, Dusticier G, Clément JL: Mechanism underlying cuticular hydrocarbon homogeneity in the ant *Camponotus vagus* (Scop) (Hymenoptera, Formicidae) - role of postpharyngeal glands. *Journal of Chemical Ecology* 1995, 21(8):1127-1148.

Mpuru S, Blomquist GJ, Schal C, Roux M, Kuenzli M, Dusticier G, Clément JL, Bagnères AG: Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*. *Insect Biochemistry and Molecular Biology* 2001, 31(2):139-155.

Pedrini N, Ortiz-Urquiza A, Huarte-Bonnet C, Fan Y, Juarez P, Keyhani NO: Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. *Proceedings of the National Academy of Sciences of the United States of America* 2015, 112(28):E3651-E3660.

Polidori C, Boesi R, Isola F, Andrietti, F: Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): the role of prey size. *European Journal of Entomology* 2005, 102:801-804.

Polidori C, Federici M, Papadia C, Andrietti F: Nest sharing and provisioning activity of females of the digger wasp, *Cerceris rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 2006, 73:55-65.

Pöppel A-K, Koch A, Kogel K-H, Vogel H, Kollewe C, Wiesner J, Vilcinskas A: Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly *Lucilia sericata*. *Biological Chemistry* 2014, 395(6):649-656.

Prendeville HR, Stevens L: Microbe inhibition by *Tribolium* flour beetles varies with beetle species, strain, sex, and microbe group. *Journal of Chemical Ecology* 2002, 28(6):1183-1190.

Ristich SS: The host relationship of a miltogrammid fly *Senotainia trilineata* (VDW). *Ohio Journal of Science* 1956, 56(5):271-274.

Rodrigues A, Carletti CD, Bueno OC, Pagnocca FC: Leaf-cutting ant faecal fluid and mandibular gland secretion: effects on microfungi spore germination. *Brazilian Journal of Microbiology* 2008, 39(1):64-67.

Ruther J, Sieben S, Schricker B: Nestmate recognition in social wasps: manipulation of hydrocarbon profiles induces aggression in the European hornet. Naturwissenschaften 2002, 89:111–114.

Singer TL: Roles of hydrocarbons in the recognition system of insects. American Zoologist 1998, 38:394-405

Soroker V, Hefetz A: Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. *Journal of Insect Physiology* 2000, 46(7):1097-1102.

Soroker V, Vienne C, Hefetz A: The postpharyngeal gland as a ‘‘Gestalt’’ organ for nestmate recognition in the ant *Cataglaphis niger*. *Naturwissenschaften* 1994, 81:510-513

Soroker V, Vienne C, Hefetz A: Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera, Formicidae). *Journal of Chemical Ecology* 1995, 21(3):365-378.

Soroker V, Fresneau D, Hefetz A: Formation of colony odor in ponerine ant *Pachycondyla apicalis*. *Journal of Chemical Ecology* 1998, 24(6):1077-1090.

Strohm E. Factors affecting body size and fat content in a digger wasp. *Oecologia* 2000, 123: 184-191.

Strohm E, Linsenmair KE: Low resource availability causes extremely male biased investment ratios in the European beewolf, *Philanthus triangulum* F (Hymenoptera, Sphecidae). *Proceedings of the Royal Society B-Biological Sciences* 1997, 264:423–9.

Strohm E, Linsenmair KE: Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 2001, 26:198-203.

Strohm E, Marliani A: The cost of parental care: Prey hunting in a digger wasp. *Behavioral Ecology* 2002, 13:52–8.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36:113–22

Strohm E, Herzner G, Kaltenpoth M, Boland W, Schreier P, Geiselhardt S, Peschke K, Schmitt T: The chemistry of the postpharyngeal gland of female European beewolves. *Journal of Chemical Ecology* 2008, 34:575–83.

Strohm E, Kaltenpoth M, Herzner G: Is the postpharyngeal gland of a solitary digger wasp homologous to ants? Evidence from chemistry and physiology. *Insectes Sociaux* 2010, 57(3):285-291.

Van den Dool H, Kratz PD: A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography* 1963, 11:463-471.

Vienne C, Soroker V, Hefetz A: Congruency of hydrocarbon patterns in heterospecific groups of ants - transfer and/or biosynthesis. *Insectes Sociaux* 1995, 42(3):267-277.

Weiss K, Parzefall C, Herzner G. Multifaceted defense against antagonistic microbes in developing offspring of the parasitoid wasp *Ampulex compressa* (Hymenoptera, Ampulicidae). *PLoS ONE* 2014, 9.

Weiss K, Strohm E, Kaltenpoth M, Herzner G: Comparative morphology of the postpharyngeal gland in the Philanthinae (Hymenoptera, Crabronidae) and the evolution of an antimicrobial brood protection mechanism. *BMC Evolutionary Biology* 2015, 15(1).

Willers D, Lehmann-Danzinger H, Führer E: Antibacterial and antimycotic effect of a newly discovered secretion from larvae of an endoparasitic insect, *Pimpla turionellae*. *Archives of Microbiology* 1982, 133:225-229.

Witte V, Foitzik S, Hashim R, Maschwitz U, Schulz S: Fine tuning of social integration by two myrmecophiles of the ponerine army ant, *Leptogenys distinguenda*. *Journal of Chemical Ecology* 2009, 35:355-367.

Yasui H, Akino T, Yasuda T, Fukaya M, Ono H, Wakamura S: Ketone components in the contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca*, and pheromonal activity of synthetic ketones. *Entomologia Experimentalis et Applicata* 2003, 107(3):167-176.

# CHAPTER 4

# DOES THE MORPHOLOGY OF THE POSTPHARYNGEAL GLAND REFLECT POTENTIAL FUNCTIONAL CHANGES IN BEE- *vs.* BEETLE-HUNTING SPECIES OF THE DIGGER WASP GENUS *CERCERIS* (HYMENOPTERA, CRABRONIDAE)?

Katharina Weiss, Erhard Strohm, Thomas Schmitt, Mareike Wurdack, Gudrun Herzner

## 4.1 Summary

Female beewolves, digger wasps of the tribe Philanthini (Hymenoptera, Philanthinae), provision their offspring with paralyzed bees and wasps in subterranean brood cells. To counter the threat of fungal infestation of the larval provisions, females of the three closely related species *Philanthus triangulum*, *Philanthus gibbosus*, and *Trachypus elongatus*, embalm their prey with unsaturated hydrocarbons (HCs) from their large and complex postpharyngeal glands (PPG), which reduces water condensation on the bee's surface and thus retards fungal growth. In the related genus *Cerceris* that shares a similar nesting behavior with beewolves, some species prey upon bees, but most species hunt beetles as larval food. It has been hypothesized that the use of bees as larval provisions might have triggered the evolution of prey embalming in beewolves, since bees might be particularly prone to fungal infestation. Supporting this view, the PPGs of beetle-hunting *Cerceris arenaria* and *Cerceris quinquefasciata* have been found to consist only of simple tubular evaginations of the pharynx, differing markedly from the complex glands of beewolves, and the PPG of *C. arenaria* contains only very low proportions of unsaturated HCs. Following the above hypothesis, it may be speculated that bee-hunting *Cerceris* employ some kind of prey embalming. If so, they might possess larger and more complex PPG than beetle-hunters, or they may have evolved some other HC storage organ. Using light-microscopy and 3D-reconstruction, we assessed head gland morphology in two bee-hunting *Cerceris* species and compared them to their beetle-hunting congeners. We found PPGs as well as mandibular glands in all species. However, we did not detect any differences in morphology and size of these glands between 'prey-types'. Thus, based on PPG morphology, we cannot conclusively assert whether the PPG is involved in any form of prey preservation in these species.

## 4.2 Introduction

Parental care is crucial for the survival of the offspring in numerous animal species (Clutton-Brock 1991). Some of the components of parental care, like nest construction or the provisioning of the brood are obvious (e.g. Strohm & Marliani 2002), but others, however, may be less conspicuous. In insects, one vitally important but often rather 'cryptic' component of parental care is the protection of the brood and/or the larval provisions against microorganisms that pose severe threats to the offspring both as pathogens (Hajek & St Leger 1994, Sikorowski et al. 2001, Secil et al. 2012) and food competitors (Janzen 1977, Burkepile et al. 2006, Rozen et al. 2008, Trienens et al. 2010).

An intriguing example for such a cryptic antimicrobial defense has been described from beewolves, solitary digger wasps of the tribe Philanthini (Hymenoptera, Crabronidae, Philanthinae). Females of the European beewolf *Philanthus triangulum* prey exclusively on honeybee workers, *Apis mellifera*, which are stored in subterranean brood cells under warm and humid conditions (e.g. Strohm & Linsenmair 2001). Prior to oviposition females extensively lick the surface of their prey (Strohm & Linsenmair 2001), thereby applying ample amounts of hydrocarbons (HCs) from their large postpharyngeal gland (PPG) (Strohm et al. 2007, Herzner et al. 2007) to the bee's cuticle (Herzner & Strohm 2007, Herzner et al. 2007, 2011a). This process, termed 'prey embalming', does not only multiply the total amount of HCs but, more importantly, also the proportion of unsaturated HCs on the bee (Herzner & Strohm 2007, Herzner et al. 2007). The resulting oily layer of unsaturated HCs reduces the condensation of water, which renders the microclimatic conditions on the bee's surface unsuitable for fungal growth (Herzner & Strohm 2007). In this way prey embalming significantly retards the decay of the larval food (Strohm & Linsenmair 2001, Herzner & Strohm 2007) and enhances larval survival (Herzner et al. 2011a). The same prey preservation mechanism has as yet also been found in two closely related species, the North American *Philanthus gibbosus* (Weiss et al. 2015) and the South American *Trachypus elongatus* (Herzner et al. 2013).

All female beewolves investigated to date possess large, complex PPGs that consist of two glove- or comb-shaped reservoirs that originate from the pharynx and occupy a considerable part of the front head capsule (Strohm et al. 2007, Herzner et al. 2013, Weiss et al. 2015), resembling the PPG of most ants in which this gland has originally been described (e.g. Soroker et al. 1995a, Lucas et al. 2004, Eelen et al. 2006), and predominantly contain unsaturated HCs (Weiss et al., *in*

*preparation*). Thus, prey embalming may be a common component of parental care in this philanthine tribe (Weiss et al. 2015, Weiss et al. *in preparation*).

Besides the tribe Philanthini, the so-called beewolves (comprising the genera *Philanthus*, *Trachypus* and *Philanthinus*), the subfamily Philanthinae includes two more tribes, the Aphilanthopsini (comprising *Aphilanthops* and *Clypeadon*) and the Cercerini (comprising *Cerceris*, *Eucerceris* and *Pseudoscolia*) (Alexander 1992, Kaltenpoth et al. 2014). Interestingly, we found marked differences in both PPG morphology and chemistry between beewolves and species of the other two tribes. The PPGs of female *Aphilanthops frigidus* and *Clypeadon laticinctus* (tribe Aphilanthopsini), as well as *Cerceris arenaria* and *Cerceris quinquefasciata* (tribe Cercerini) consist of simple tubular evaginations of the pharynx, thus differing markedly from the large, complex glands of beewolves (Weiss et al. 2015). Moreover, the PPG secretion of *C. laticinctus* and *C. arenaria* contains only lower proportions of unsaturated HCs and also in *A. frigidus* unsaturated compounds account for a smaller share of the total secretion as compared to the Philanthini (Weiss et al., *in preparation*). These findings raise the question whether prey embalming has only evolved in beewolves.

All members of the subfamily Philanthinae share basic life-history traits, including nest construction and provisioning (e.g. Evans 1962, 1971, 2000, Bohart & Menke 1976, Evans & O'Neill 1988, Polidori et al. 2005, 2006), but there are considerable differences in prey spectrum between the tribes. Female Philanthini hunt for bees and sometimes other aculeate wasps (e.g. Evans & O'Neill 1988) and Aphilanthopsini hunt exclusively on ants (Ristich 1956, Evans 1962). While some Cercerini likewise hunt on solitary bees (e.g. Bohart & Menke 1976), most species of the genus *Cerceris*, however, provision beetles (mainly weevils and buprestids, e.g. Bohart & Menke 1976), including the two *Cerceris* species for which morphology (*C. arenaria* and *C. quinquefasciata*, Weiss et al. 2015) and chemistry (*C. arenaria*; Weiss et al., *in preparation*) of the PPG are known. Hence, one possible factor in the evolution of prey embalming in the Philanthinae might be differences in the microbial load and the general susceptibility to fungal infestation of the different prey types, with bees likely being the most risky food source (see discussion in Weiss et al. 2015). Thus, it may be hypothesized that only in bee-hunting species the benefits of prey embalming outweighed its costs (Herzner et al. 2011a). In this regard, the genus *Cerceris* may provide especially interesting insights, since one might expect bee-hunting *Cerceris* to exhibit some kind of prey preservation mechanism similar to beewolves. Unfortunately, nothing is known about the PPGs of bee-hunting *Cerceris* species.

To contribute a further piece of the puzzle whether prey embalming as an antimicrobial parental investment is restricted to the Philanthini, or has also evolved in other philanthine taxa, we compared females of the two bee-hunting species *Cerceris sabulosa* and *Cerceris rybyensis* and the two previously investigated beetle-hunters *C. arenaria* and *C. quinquefasciata* (Weiss et al. 2015) by light microscopy of semithin histological sections and 3D-reconstructions based on these sections. We assessed the occurrence, size, and morphology of the PPGs and any other head glands between the two groups of *Cerceris* and discuss our findings with regard to possible differences in prey preservation. The presence of more complex and/or larger PPGs in bee-hunting *Cerceris* than in their beetle-hunting congeners or the evolution of some other possible HC storage organ not occurring in beetle-hunters would point to the existence of some kind of prey embalming mechanism in bee-hunting *Cerceris*.

## 4.3 Methods

### 4.3.1 Specimens

Females of the two beetle-hunting species *C. arenaria* and *C. quinquefasciata* and the two bee-hunting species *C. rybyensis* and *C. sabulosa* were collected from field populations. *C. sabulosa* and *C. quinquefasciata* were collected at Kaiserstuhl near Freiburg, Germany, *C. rybyensis* was collected in Nuremberg, Germany, and *C. arenaria* were collected near Pula, Croatia.

### 4.3.2 Histology

Insects were anesthetized with carbon dioxide and decapitated. Heads of *C. sabulosa* (N=3), *C. quinquefasciata* (N=3) and *C. arenaria* (N=2) were fixed in formalin-ethanol-acetic acid after Scheuring, the head of a *C. rybyensis* female (N=1) was fixed in alcoholic Bouin (Böck 1989). Both compound eyes were laterally cut off using razor blades to facilitate the infiltration of the embedding medium. After fixation, heads were rinsed in 80% ethanol and dehydrated in a graded ethanol series and propylene oxide. Finally, they were embedded in Epon 812 (Polysciences Europe GmbH, Eppelheim, Germany) according the suppliers instructions. Sagittal semithin sections (4 µm) were cut with a microtome (Reichert Ultracut, Leica Microsystems AG, Wetzlar, Germany) equipped with a diamond knife and stained with toluidine blue (Adam & Czihak 1964). The resulting series of histological sections were examined under a light microscope (Zeiss

Axiophot 2, Carl Zeiss Microscopy GmbH, Jena) and used for 3D-reconstructions of the head glands.

### 4.3.3 3D-Reconstruction

To visualize shape and location of head glands, 3D-reconstructions of the gland reservoirs as well as basic structures within the head capsule (brain, pharynx, and ocelli) were generated for one female of each of the four investigated species. For 3D-reconstruction, continuous series of semithin sections were photographed using a digital microscope camera (Olympus DP20; Olympus, Hamburg, Germany) attached to a light microscope (Zeiss Axiophot 2, Zeiss GmbH, Jena, Germany) using 2.5x or 5x PlanNeofluar objectives. The digitalized images were automatically aligned to each other using the software package TrakEM2 (Cardona et al. 2012) for the image processing software Fiji (Schindelin et al. 2012); the alignments were manually corrected where necessary. The outer margin of the epithelium surrounding the reservoirs of the MG and the PPG, as well as other relevant structures within the head capsule were then marked as 3D-objects in TrakEM2 by manually outlining them in each picture of a series. 3D-reconstructions were then calculated and visualized using Fiji's 3D-viewer plug-in (Schmid et al. 2010).

### 4.3.4 Comparison of gland volumes

Our investigations revealed the presence of PPGs and mandibular glands (MG) (see Results). To assess possible differences in gland size among bee- and beetle-hunting *Cerceris* species, gland volumes were compared among those two groups. Therefore, 3D-reconstructions of the PPGs and MGs of the bee-hunting *C. sabulosa* (N=3) and *C. rybyensis* (N=1) and the beetle-hunting *C. arenaria* (N=2) and *C. quinquefasciata* (N=3) were made as described above. In order to exclude allometric effects, relative gland volumes in relation to brain volume were compared. The relative PPG volume for each individual was calculated as the ratio of PPG volume to brain volume [nl/nl]. Relative gland volumes were compared by exact Mann-Whitney *U* tests using the statistics software package PAST (Version 2.08b, Hammer et al. 2001). Owing to the small sample size and the rather large variance in MG size within bee- and beetle-hunters, respectively, a statistical comparison of MG volume was unfortunately not possible. We therefore provide only descriptions of these glands.

## 4.4 Results

### 4.4.1 Overall morphology of head glands

Females of all four investigated *Cerceris* species possess a gland reservoir in the front part of their head capsule (Figure 4.1). We found no obvious differences in the morphology of this reservoir between species, thus its general appearance is further described without referring to a particular species. The gland reservoir consists of an upper and a lower part which each comprise two tube-shaped evaginations of the pharynx (Figure 4.2 A). Its origin from the pharynx at the posterior part of the hypopharyngeal plate classifies this gland as a PPG. The two upper tubes of the PPG originate from the dorsal side of the pharynx and extend laterally with their distal ends tending somewhat backwards over the brain. The two lower tubes originate ventrally from the pharynx and extend laterally towards the compound eyes. The diameter of the gland tubes is variable and seems to depend on the degree of filling, in the specimens used for the 3D-reconstructions it ranges between 20 - 75 µm for the upper tubes and 50 - 120 µm for the lower tubes. In all investigated species the lower part of the gland is the more voluminous one, accounting for approximately 60 to 65 % of the total PPG volume.

A second set of glands was found laterally on both sides of the head capsule that open to the upper side of the mandibular base and were thus identified as MGs. In *C. arenaria* and *C. quinquefasciata* an extension of the MG reservoir proceeds medially towards the pharynx (Figure 4.1 A and B). This extension is much less pronounced in *C. sabulosa* (Figure 4.1 C) and missing in *C. rybyensis* (Figure 4.1 D). The MGs of all species are associated with gland cells (Figure 4.1, see also section 4.4.2), the beetle-hunters *C. arenaria* and *C. quinquefasciata* seem to possess considerably more of these gland cells, however (see below).

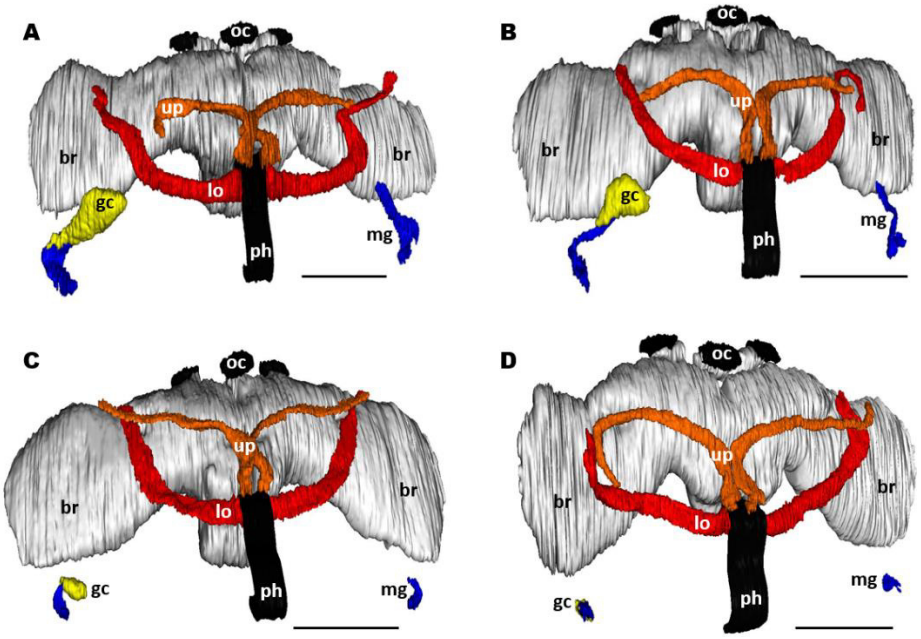


**Figure 4.1** 3D-reconstructions of the postpharyngeal glands (PPG) and the mandibular glands (MG) of female *Cerceris*. **(A)** *Cerceris arenaria*, **(B)** *Cerceris quinquefasciata*, **(C)** *Cerceris sabulosa*, **(D)** *Cerceris rybyensis*. Note that the gland cells associated with the MG are only shown on the right body side. In (D) these gland cells are hardly visible due to the small size of the gland and the orientation of the image. Abbreviations: br, brain (light grey); gc, gland cells (yellow); lo, lower tubes of the PPG reservoir (red); mg, reservoir of the MG (blue); oc, ocelli (black); ph, pharynx (black); up, upper tubes of PPG reservoir (orange). Scale bars = 0.5 mm.

### 4.4.2 Light-microscopy

As the light-microscopic examination of semithin histological sections did not reveal species-specific differences, the fine-structure of the head glands detected in *Cerceris* females is described without reference to a particular species. The walls of both upper and lower part of the PPG are formed by a monolayered epithelium (Figure 4.2). The epithelial cells bear short hairs (1-5 hairs per cell) on their apical side which resemble the hairs found on the epithelial cells of the pharynx (Figure 4.2). However, the distribution of these hairs is not consistent within and among the different gland tubes of an individual. Generally, there are more epithelial hairs in the upper tubes compared to the lower tubes of the gland (Figure 4.2 B and C). Furthermore, their density declines somewhat from the gland opening at the pharynx to the distal tip of the tubes and there are also cells that do not bear any hairs. The PPG reservoir of female *Cerceris* is not associated with any gland cells and, although there are muscle fibers surrounding the PPG reservoir near the opening to the pharynx (Figure 4.2 A), they do not seem to be directly associated with the gland.

The MG generally consists of a monolayered epithelium which does not bear any conspicuous hairs, but instead, the walls of the MG show small folds (Figure 4.3). The MGs of all four

investigated species are associated with gland cells. In *C. quinquefasciata* and *C. arenaria* roughly 130 - 170 class 1 and class 3 gland cells (defined after Noirot & Quennedey 1974) closely surround the extension of the gland reservoir (Figure 4.3 A and B). In *C. sabulosa* the number of associated gland cells is smaller (about 50 cells, Figure 4.3 C) and the MG of the investigated *C. rybyensis* specimen was only associated with about 20 scattered class 1 gland cells (Figure 4.1 D).

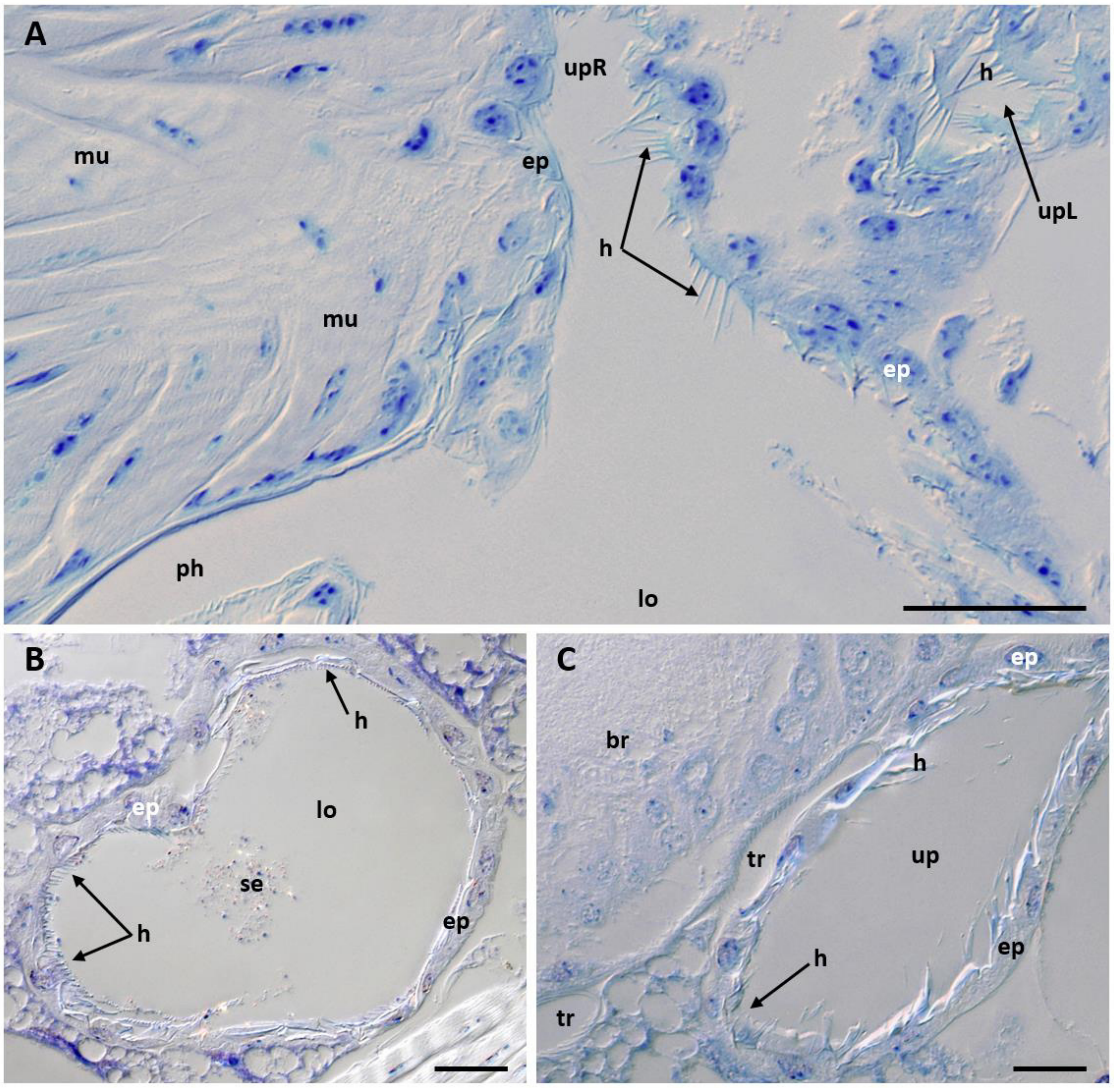


**Figure 4.2** Histological sections of the postpharyngeal glands (PPG) of female *Cerceris*. **(A)** Openings of a lower (lo) and the right upper tube (upR) of the PPG to the pharynx in *Cerceris sabulosa*; the profile of the left upper tube of the PPG (upL) approaching the pharynx can also be seen; scale bar = 25 µm. **(B)** Profile of a lower PPG tube of *Cerceris rybyensis*, with some secretion visible in the lumen of the reservoir; scale bar = 10 µm. **(C)** Profile of an upper PPG tube of *Cerceris rybyensis* passing the brain; scale bar = 10 µm. Abbreviations: b, brain; ep, epithelium of the PPG; h, epithelial hairs; lo, lumen of the lower part of the PPG; mu, muscle fibers; ph, pharynx; se, secretion within the PPG reservoir; tr, tracheole; up, lumen of the upper part of the PPG (upR, right PPG tube; upL, left PPG tube).

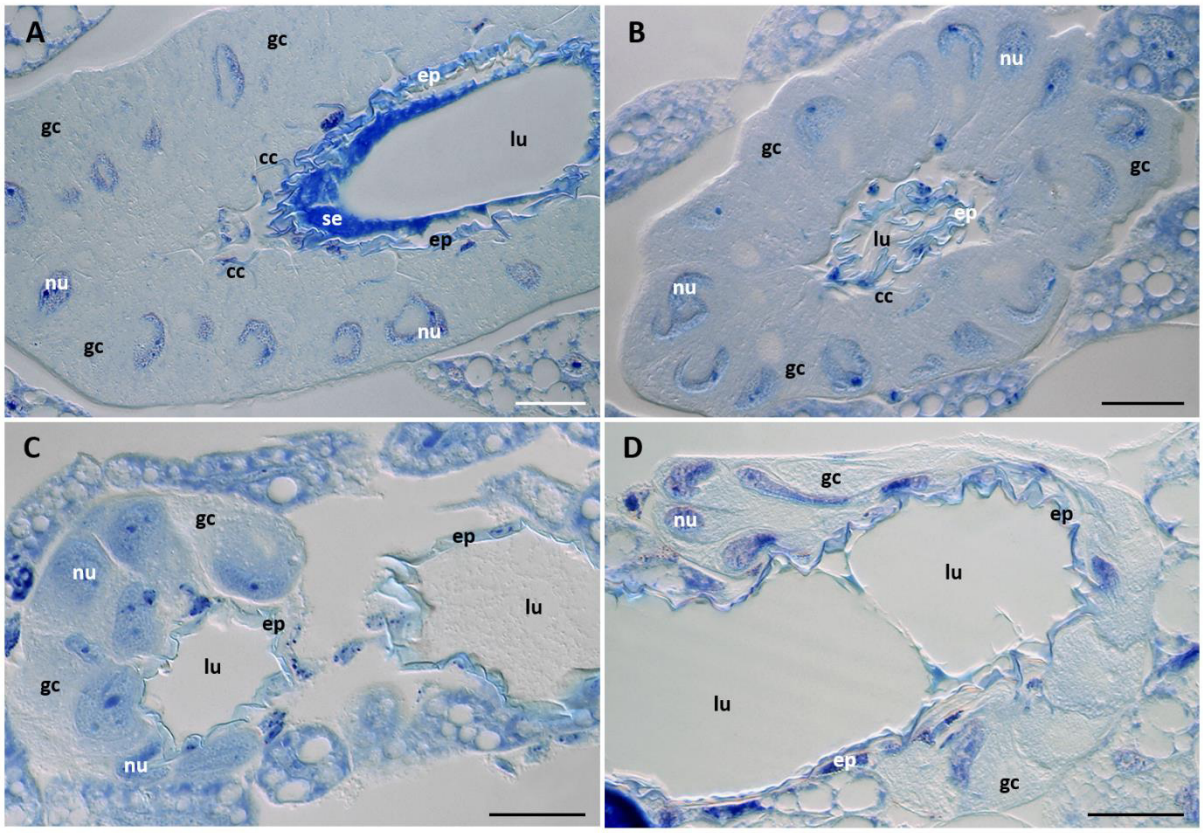


**Figure 4.3** Histological sections of the mandibular glands (MG) and the associated gland cells of female *Cerceris*. **(A)** *Cerceris quinquefasciata*, note the secretion adhering to the inner wall of the reservoir, **(B)** *Cerceris arenaria*, **(C)** *Cerceris sabulosa*, **(D)** *Cerceris rybyensis*. Abbreviations: cc, conducting canal of class 3 gland cell; cu, cuticle; ep, epithelium of the MG reservoir; gc, gland cells; lu, lumen of the MG reservoir; nu, nucleus of gland cell; se, secretion adhering to the epithelium of the reservoir. Scale bars = 25 µm.

### 4.4.3 Comparison of gland volumes

The total PPG volumes were 2.5±0.2 nl (median ± median absolute deviation) for *C. quinquefasciata* (N=3), 7.3±3.2 nl for *C. arenaria* (N=2), and 2.7±0.4 nl for *C. sabulosa* (N=3). The volume of the PPG of the *C. rybyensis* specimen was 7.1 nl (N=1). The comparison of the relative gland volumes (in relation to brain volume, [nl/nl]) between the bee-hunters *C. sabulosa* and *C. rybyensis* (0.4±0.1, N=4) and the beetle-hunters *C. quinquefasciata* and *C. arenaria* (0.4±0.1, N=5) revealed no significant difference (Mann-Whitney *U* test: $U = 10$, exact p=1).

## 4.5 Discussion

Our results clearly show that females of *C. arenaria*, *C. quinquefasciata*, *C. rybyensis*, and *C. sabulosa* possess a gland reservoir in the frontal part of their head capsule that qualifies as a PPG due to its origin from the posterior part of the pharynx. Its basic morphology resembles the PPGs described in other taxa of the subfamily Philanthinae (Strohm et al. 2007, Herzner et al. 2013, Weiss et al. 2015), and ants (e.g. Soroker et al. 1995a, Lucas et al. 2004, Eelen et al. 2006). In the four investigated *Cerceris* species, as in all other Philanthinae (Strohm et al. 2007, Herzner et al. 2013, Weiss et al. 2015), the females' PPGs consist of lateral/dorsal evaginations of the pharynx that are located anterior to the brain. Furthermore, in all these taxa the walls of the reservoir consist of a monolayered epithelium, the cells of which bear hairs on their apical sides reaching into the lumen of the reservoir. There are no gland cells associated with the PPG in any of the above species, suggesting that the gland contents are produced elsewhere and subsequently sequestered into the PPG reservoir (Strohm et al. 2007, 2008, 2010, Herzner et al. 2013, Weiss et al. 2015).

We found no differences in PPG size or morphology among bee- and beetle-hunting *Cerceris*. In all four species both upper and lower parts consist of simple paired tubes without any branches, thus resembling members of the Aphilanthopsini but differing markedly from beewolves (Weiss et al. 2015). In the later, the upper part of the PPG shows a complex glove- or comb-like structure with a number of 'fingers' branching of from a common root on both sides of the pharynx, whereas the lower part is formed by an unpaired sac-like evagination which is considerably smaller than the upper parts (Strohm et al. 2007, Herzner et al. 2013, Weiss et al. 2015). Furthermore, the volume of the PPG in *Cerceris* is much smaller than in beewolves ranging between 2.5 and 7.3 nl, depending on the species. In the European beewolf *P. triangulum*, the volume of the PPG has been estimated to be 3-4 µl (Strohm et al. 2007) and in *Trachypus* it ranges between 0.1 µl for *T. boharti* and 0.27 µl for *T. elongatus* (Herzner et al. 2013). Thus, the volume of the PPG of *Cerceris* is roughly 1/1000 of the PPG of *P. triangulum* and 1/100 of the PPG of *Trachypus*. Even though *P. triangulum* (average head capsule width 4.25-4.5 mm, Strohm & Linsenmair 1997) and the two *Trachypus* species (*T. boharti*: 3.4±0.2 mm, *T. elongatus*: 3.6±0.1 mm, Herzner et al. 2013) are on average somewhat larger than *Cerceris* (e.g. *C. sabulosa:* 2.7±0.1 mm, N=3; *C. quinquefasciata:* 2.6±0.1 mm, N=3) these moderate differences in body size cannot account for the large differences in gland volume.

As indicated by an ancestral state reconstruction of the shape of the PPG within the Philanthinae using *Ampulex compressa*, the most basal apoid taxon for which a PPG has been described (Herzner et al. 2011b) as outgroup (Weiss et al. 2015), it is reasonable that the small tube-shaped PPGs of Cercerini and Aphilanthopsini are the evolutionary precursors of the large and branched PPGs of beewolves. The PPG of *A. compressa* resembles the gland of Cercerini and Aphilanthopsini in that it consists of two small tubular evaginations of the pharynx (though in *A. compressa* the PPG is located behind the brain) (Herzner et al. 2011b).

In the three beewolf species known to embalm their prey with unsaturated HCs as an antimicrobial strategy, *P. triangulum*, *P. gibbosus*, and *T. elongatus* (Strohm & Linsenmair 2001, Herzner et al. 2011a, 2013, Weiss et al. 2015), the PPG functions as the reservoir for the embalming secretion (Herzner et al. 2007, 2013, Strohm et al. 2008, Weiss et al. 2015). The chemical composition of the females' PPG secretion in the Cercerini, however, has thus far only been investigated for the beetle-hunting *C. arenaria*. In this species, the PPG contains only a comparatively small proportion of unsaturated HCs, but is dominated by methyl-branched HCs (Weiss et al., *in preparation*). Thus, the occurrence of only small, simple PPG and the lack of unsaturated HCs together make it unlikely that female *C. arenaria* employ prey embalming as described for beewolves, which would support the hypothesis that the use of non-Hymenopteran prey may make antimicrobial brood care unnecessary (Weiss et al. 2015). Yet, with no evidence that beetle-hunting *Cerceris* do embalm their prey, our findings raise the questions why they possess PPGs at all? One possible answer may be that the PPG serves other functions apart from prey embalming. The generally close resemblance between the HC-profiles of the PPG and the cuticle (Bagnères & Morgan 1991, Akino et al. 2004, Lucas et al. 2004, Strohm et al. 2010, Herzner et al. 2011b, 2013), suggests that the PPG is involved in the storage and distribution of cuticular HCs, as has been demonstrated in ants (Soroker et al. 1994, 1995b, Lenoir et al. 1999). For *A. compressa*, which does not embalm its prey, the gland has been proposed to serve simply as a storage organ for cuticular HC (Herzner et al. 2011b). Being one station along the HC pathway may explain the presence of the PPG in beetle-hunting *Cerceris* species that do not embalm their prey.

If, on the other hand, bee-hunting *Cerceris* do employ some kind of prey embalming with PPG secretion, the above reasoning would imply that they should possess larger PPG and/or higher proportions of unsaturated compounds than their beetle-hunting congeners. Our results on PPG morphology, thus, contradict this hypothesis, since we found no differences in PPG morphology or size among both groups of *Cerceris*. Then again, since we have no knowledge about the chemical composition of the PPG content of bee-hunting *Cerceris*, we cannot yet reject the hypothesis that

they do embalm their prey. For instance, it seems plausible that the optimal quantities of HCs applied to the prey as a protection against mold growth depend on various abiotic and biotic factors, like temperature and humidity inside the nest, or surface chemistry and topography, as well as microbe load of the prey (Herzner & Strohm 2007, Herzner et al. 2013, Weiss et al. 2015). These factors are likely to determine the optimum amount and composition of HCs required for effective prey preservation and will thus select for a particular gland size, morphology and content over evolutionary times. It might well be that bee-hunting *Cerceris* adds only small quantities of hydrocarbons to the bees and that their small PPG therefore suffices as HC reservoir. *T. elongatus* females, for example, coat their prey with lower amounts of HCs and also possess smaller PPGs than *P. triangulum* (Herzner et al. 2013). Furthermore, the size and gross morphology of the gland might not reflect its quality or effectivity as HC source for prey embalming. The epithelium of the PPG might be highly active and allow for a faster sequestration and secretion of HCs. To evaluate this possibility studies on the ultrastructure of the PPG epithelia of *Cerceris* are needed.

Alternatively, in bee-hunting *Cerceris* another gland might serve as the source of a potential embalming secretion. The only other head gland detected in this study was the MG. Even though the MG has long been identified in *Cerceris* (Ågren 1977), its function as well as the chemistry of its contents have to date not been described in this genus. For the stingless bee *Melipona quadrifascitata*, however, a contribution of MG-derived HCs to the surface chemistry of the cuticle has been suggested (Cruz-Landim et al. 2012). If the MG were the source of the embalming secretion it should be larger or more complex in the bee-hunting species. The opposite seems to be the case, however. Generally, the MGs are small compared to the PPGs in all investigated *Cerceris* species, and they are more complex and associated with more gland cells in the beetle-hunting *Cerceris*. These observations make an involvement of the MG in prey embalming unlikely.

## 4.6 Conclusion

Bee-hunting *Cerceris* have a nesting behavior similar to the closely related beewolves (Evans 1971, 2000, Bohart & Menke 1976, Polidori et al. 2005, 2006). They choose prey that might be susceptible to mold growth (see discussion in Weiss et al. 2015). Our finding that bee-hunting *Cerceris* females possess PPGs supports the hypothesis that they might exhibit a parental care behavior similar to beewolves, which fight fungal degradation of the larval provisions by embalming them with HCs from their PPG to enhance offspring survival. The prediction that these PPGs should be larger or more complex as compared to the glands of beetle-hunters (Weiss et al. 2015), which, according to the data available, do probably not embalm their prey, could not be confirmed, however. All *Cerceris* investigated here possess PPGs of similar overall appearance and size. Based on these results we can therefore not conclusively assert that in *Cerceris* the PPG is involved in the application of HCs to prey bees. A broad-scale chemical analysis of the PPG secretion in bee-hunting, and further beetle-hunting *Cerceris*, as well as the surface chemistry of provisioned prey items from both 'prey-types' seems appropriate to further clarify the phylogenetic distribution of prey embalming and the selection pressures shaping this 'cryptic' component of parental care in the Philanthinae.

## 4.7 References


Adam H, Czihak G: Arbeitsmethoden der makroskopischen und mikroskopischen Anatomie. Stuttgart: Gustav Fischer Verlag; 1964.

Ågren L: Mandibular gland morphology of *Cerceris rybyensis* (L), (Hymenoptera: Philanthidae). *Zoon* 1977, 5(2):91-95.

Akino T, Yamamura K, Wakamura S, Yamaoka R: Direct behavioral evidence for hydrocarbons as nestmate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Applied Entomology and Zoology* 2004, 39(3):381-387.

Alexander BA: A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 1992, 17(2):91-108.

Bagnères AG, Morgan ED: The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 1991, 47(1):106-111.

Böck P: Romeis mikroskopische Technik. 17th edition. München, Wien, Baltimore: Urban und Schwarzenberg; 1989.

Bohart RM, Menke AS: Sphecid wasps of the world: A generic revision. University of California Press; 1976.

Burkepile DE, Parker JD, Woodson CB, Mills HJ, Kubanek J, Sobecky PA, Hay ME: Chemically mediated competition between microbes and animals: Microbes as consumers in food webs. *Ecology* 2006, 87(11):2821-2831.

Cardona A, Saalfeld S, Schindelin J, Arganda-Carreras I, Preibisch S, Longair M, Tomancak P, Hartenstein V, Douglas RJ: TrakEM2 software for neural circuit reconstruction. *Plos One* 2012, 7(6):e38011.

Clutton-Brock TH: The evolution of parental care. 1st edition. Princeton: Princeton University Press; 1991.

Cruz-Landim Cd, Ferreira-Caliman MJ, Gracioli-Vitti LF, Zucchi R: Correlation between mandibular gland secretion and cuticular hydrocarbons in the stingless bee *Melipona quadrifasciata*. *Genetics and Molecular Research* 2012, 11:966-977.

Eelen D, Borgesen L, Billen J: Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 2006, 87(2):101-111.

Evans HE: A review of nesting behavior of digger wasps of the genus *Aphilanthops*, with special attention to the mechanism of prey carriage. *Behaviour* 1962, 19:239-260.

Evans HE: Observations on the nesting behavior of wasps of the tribe Cercerini. *Journal of the Kansas Entomological Society* 1971, 44(4):500-523.

Evans HE: Observations on the biology of *Cerceris mimica* Cresson (Hymenoptera: Sphecidae: Philanthinae). *Journal of the Kansas Entomological Society* 2000, 73(4):220-224.

Evans HE, O' Neill KM: The natural history and behavior of North American beewolves. 1st edition. Ithaca: Cornell University Press; 1988.

Hajek AE, St Leger RJ: Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* 1994, 39:293-322.

Hammer Ø, Harper DAT, Ryan PD: PAST: Paleontological statistics software package for education and data analysis. *Paleontologica Electronica* 2001, 4.

Herzner G, Strohm E: Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007, 17(2):R46-R47.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007, 33(4):849-859.

Herzner G, Engl T, Strohm E: Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011a, 82(2):321-328.

Herzner G, Ruther J, Goller S, Schulz S, Goettler W, Strohm E: Structure, chemical composition and putative function of the postpharyngeal gland of the emerald cockroach wasp, *Ampulex compressa* (Hymenoptera, Ampulicidae). *Zoology* 2011b, 114(1):36-45.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. *Plos One* 2013, 8(12):e82780.

Janzen DH: Why fruits rot, seeds mold, and meat spoils. *American Naturalist* 1977, 111(980):691-713.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111:6359–64.

Lenoir A, Fresneau D, Errard C, Hefetz A: Individuality and colonial identity in ants: The emergence of the social representation concept. In: *Information processing in social insects.* Springer; 1999: 219-237.

Lucas C. Pho D, Fresneau D, Jallon JM: Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*. *Journal of Insect Physiology* 2004, 50:595-607.

Noirot C, Quennedey A: Fine-structure of insect epidermal glands. *Annual Review of Entomology* 1974, 19:61-80.

Polidori C, Boesi R, Isola F, Andrietti F: Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): The role of prey size. *European Journal of Entomology* 2005, 102(4):801-804.

Polidori C, Federici M, Papadia C, Andrietti F: Nest sharing and provisioning activity of females of the digger wasp, *Cerceiis rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 2006, 73(1):55-65.

Ristich SS: The host relationship of a miltogrammid fly *Senotainia trilineata* (VDW). *Ohio Journal of Science* 1956, 56(5):271-274.

Rozen DE, Engelmoer DJP, Smiseth PT: Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences of the United States of America* 2008, 105:17890-17895.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A: Fiji: An open-source platform for biological-image analysis. *Nature Methods* 2012, 9(7):676-682.

Schmid B, Schindelin J, Cardona A, Longair M, Heisenberg M: A high-level 3D visualization API for Java and ImageJ. *BMC Bioinformatics* 2010, 11.

Secil ES, Sevim A, Demirbag Z, Demir I: Isolation, characterization and virulence of bacteria from *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Biologia* 2012, 67(4):767-776.

Sikorowski PP, Lawrence AM, Inglis GD: Effects of *Serratia marcescens* on rearing of the tobacco budworm (Lepidoptera: Noctuidae). *American Entomologist* 2001, 47(1):51-60.

Soroker V, Vienne C, Hefetz A, Nowbahari E: The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 1994, 81(11):510-513.

Soroker V, Hefetz A, Cojocaru M, Billen J, Franke S, Francke W: Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 1995a, 20(4):323-329.

Soroker V, Vienne C, Hefetz A: Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera, Formicidae). *Journal of Chemical Ecology* 1995b, 21, 365-378.

Strohm E, Linsenmair KE: Female size affects provisioning and sex allocation in a digger wasp. *Animal Behaviour* 1997, 54, 23-34.

Strohm E, Linsenmair KE: Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 2001, 26(2):198-203.

Strohm E, Marliani A: The cost of parental care: Prey hunting in a digger wasp. *Behavioral Ecology* 2002, 13(1):52-58.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36(2):113-122.

Strohm E, Herzner G, Kaltenpoth M, Boland W, Schreier P, Geiselhardt S, Peschke K, Schmitt T: The chemistry of the postpharyngeal gland of female European beewolves. *Journal of Chemical Ecology* 2008, 34(5):575-583.

Strohm E, Kaltenpoth M, Herzner G: Is the postpharyngeal gland of a solitary digger wasp homologous to ants? Evidence from chemistry and physiology. *Insectes Sociaux* 2010, 57(3):285-291.

Trienens M, Keller NP, Rohlfs M: Fruit, flies and filamentous fungi - Experimental analysis of animal-microbe competition using *Drosophila melanogaster* and *Aspergillus* mould as a model system. *Oikos* 2010, 119(11):1765-1775.

Weiss K, Strohm E, Kaltenpoth M, Herzner G: Comparative morphology of the postpharyngeal gland in the Philanthinae (Hymenoptera, Crabronidae) and the evolution of an antimicrobial brood protection mechanism. *BMC evolutionary biology* 2015, 15(1).

# CHAPTER 5

## SEXUAL SELECTION AND THE EVOLUTION OF MALE PHEROMONE GLANDS IN PHILANTHINE WASPS (HYMENOPTERA, CRABRONIDAE)

*BMC Evolutionary Biology (2017), doi: 10.1186/s12862-017-0963-6*

Katharina Weiss, Gudrun Herzner, Erhard Strohm

## 5.1 Summary

**Background**

Sexual selection is thought to promote evolutionary changes and diversification. However, the impact of sexual selection in relation to other selective forces is difficult to evaluate. Male digger wasps of the tribe Philanthini (Hymenoptera, Philanthinae) scent mark territories to attract receptive females. Consequently, the organs for production and storage of the marking secretion, the mandibular gland (MG) and the postpharyngeal gland (PPG), are subject to sexual selection. In female Philanthini, these glands are most likely solely subject to natural selection and show very little morphological diversity. According to the hypothesis that sexual selection drives interspecific diversity, we predicted that the MG and PPG show higher interspecific variation in males than in females. Using histological methods, 3D-reconstructions, and multivariate statistical analysis of morphological characters, we conducted a comparative analysis of the MG and the PPG in males of 30 species of Philanthini and three species of the Cercerini and Aphilanthopsini, two related tribes within the Philanthinae.

**Results**

We found substantial interspecific diversity in gland morphology with regard to gland incidence, size, shape and the type of associated secretory cells. Overall there was a phylogenetic trend: Ensuing from the large MGs and small PPGs of male Cercerini and Aphilanthopsini, the size and complexity of the MG was reduced in male Philanthini, while their PPG became considerably enlarged, substantially more complex, and associated with an apparently novel type of secretory cells. In some clades of the Philanthini the MG was even lost and entirely replaced by the PPG. However, several species showed reversals of and exceptions from this trend. Head gland morphology was significantly more diverse among male than among female Philanthinae.

**Conclusion**

Our results show considerable variation in male head glands including the loss of an entire gland system and the evolution of a novel kind of secretory cells, confirming the prediction that interspecific diversity in head gland morphology is higher in male than in female Philanthini. We discuss possible causes for the remarkable evolutionary changes in males and we conclude that this high diversity has been caused by sexual selection.

## 5.2 Introduction

Ever since Charles Darwin introduced sexual selection as a distinct evolutionary force (Darwin 1859, 1871), its importance relative to other evolutionary processes has been debated (Huxley 1938, van Doorn et al. 2009, Cornwallis & Uller 2010, Maan & Seehausen 2011, Weissing et al. 2011, Wagner et al. 2012, Safran et al. 2013, Scordato et al. 2014). In particular, the potential of sexual selection as a driving force for speciation has received much attention (West-Eberhard 1983, Higashi 1999, Ritchie 2007, Panhuis et al. 2001, van Doorn et al. 2004). Generally, sexual selection is assumed to promote rapid evolutionary change and population divergence (Seehausen & van Alphen 1999, Hosken & Stockley 2004, Møller & Szép 2005, Arnegard et al. 2010, Kraaijeveld et al. 2011, Wagner et al. 2012, Seddon et al. 2013, Bacquet et al. 2015; but see e.g. Huxley 1938, van Doorn et al. 2004) due to different mechanisms like the Fisher-Zahavi processes (Prum 1997, Kokko et al. 2002, 2003) and sexual antagonism (Holland & Rice 1998). However, as outlined by Panhuis et al. (2001), observed diversity in a trait presumably under sexual selection may also have been caused by other evolutionary forces like natural selection, genetic drift, or mutation. Hence, one major problem in the study of sexual selection is the assessment of its effect relative to other potential causes of evolutionary change (Panhuis et al. 2001, Hosken & House 2011).

Whereas the evolution of visual and acoustic courtship signals and their structural basis have been studied extensively (e.g. Ord & Martins 2006, Garamszegi et al. 2005), the glands involved in the production of sex pheromones have received comparatively little attention (Symonds & Elgar 2008) although chemical communication is probably the oldest and predominant mode of communication in most animal taxa (Johansson & Jones 2007). Here we test the hypothesis that head glands of male digger wasps that are subject to sexual selection show higher interspecific diversity than the same glands in females, where they are under natural selection.

The mandibular glands (MG) and the postpharyngeal glands (PPG) of the solitary digger wasp subfamily Philanthinae (Hymenoptera, Crabronidae) are an excellent model system to study the relative contribution of sexual selection to evolutionary change since these glands occur in both sexes but are subject to different selection regimes in males and females. The Philanthinae consist of eight genera, separated into three tribes (Alexander 1992): the Cercerini (comprising the three genera (*Cerceris* + *Eucerceris*) + *Pseudoscolia*), the Aphilanthopsini (comprising *Clypeadon* and *Aphilanthops*), and the Philanthini, the so-called beewolves (comprising (*Philanthus* + *Trachypus*) + *Philanthinus,* with *Trachypus* most probably being a subgenus of *Philanthus*; Alexander 1992),

Kaltenpoth et al. 2014). The members of the subfamily largely share basic life-history characters, in particular with regard to female nesting behavior (e.g. Evans 1962, 1971, Bohart & Menke 1976, Evans & O'Neill 1988, Evans 2000, Polidori et al. 2005, 2006) and male reproductive behavior (e.g. Alcock 1975, Evans & O'Neill 1985, 1988, Strohm 1995, Strohm & Lechner 2000, Clarke et al. 2001, O'Neill 2001, Kroiss et al. 2010).

As best documented for the genus *Philanthus*, males establish small territories in the vicinity of female nesting aggregations (e.g. Evans & O'Neill 1988, Strohm 1995, Strohm & Lechner 2000, Kroiss et al. 2010) and scent-mark their territories with a secretion from their large head glands to attract receptive females (e.g. Schmidt et al. 1985, Evans & O'Neill 1988, Schmitt et al. 2003, Kroiss et al. 2006). Scent marking and territoriality is also known from males of some species of the tribe Cercerini (Alcock 1975, Evans & O'Neill 1985, Clarke et al. 2001, O'Neill 2001) and at least two species of the Aphilanthopsini (Alcock 1975, O'Neill 2001). Earlier publications on *Philanthus* assumed that the males' marking secretion is produced and stored in the MG (reviewed in Evans & O'Neill 1988). In the European beewolf *Philanthus triangulum* the marking secretion is in fact most likely synthesized in the gland cells of the MG (Goettler & Strohm 2008), but the main storage organ is the remarkably enlarged PPG (Kroiss et al. 2006, Herzner et al. 2007a). The MG and the PPG together are considerably larger than the brain. The huge size of the glands and the tremendous amounts of marking secretion that are produced and stored (Schmitt et al. 2003, Kroiss et al. 2006) clearly illustrate the importance of these glands for beewolf males. Moreover, there is evidence that females prefer larger males that produce and store larger amounts of pheromone in their glands and apply more secretion to their territories (Strohm et al., *unpublished data*).

In addition to the quantity of the marking secretion, its composition likely plays a decisive role for male attractiveness. In *P. triangulum*, the composition of the males' marking secretion has presumably been influenced by a female sensory bias (Herzner et al. 2005, Schmitt et al. 2007, Steiger et al. 2010). Female *P. triangulum* use (*Z*)-11-eicosen-1-ol as a kairomone to identify their only prey, honeybee workers (*Apis mellifera*), and have evolved a high sensitivity for this compound (Herzner et al. 2005). Males exploit this pre-existing female sensory bias to increase their territories' conspicuousness to females by using (*Z*)-11-eicosen-1-ol as the major component of their marking secretion (Schmitt et al. 2003, Kroiss et al. 2006, Herzner et al. 2005). Taken together, these findings imply that both the amount and the composition of the marking pheromone are important determinants of male reproductive success. Consequently, the

secretory cells that produce the marking secretion and the gland reservoirs that store it are subject to strong sexual selection.

Female Philanthinae also possess an MG and a PPG (Ågren 1977, Strohm et al. 2007, Herzner et al. 2013, Weiss et al. 2015). Females of this subfamily mass-provision subterranean brood cells with paralyzed insects as food for their progeny (e.g. Evans 1962, 1971, Evans & O'Neill 1988, Strohm & Linsenmair 1999). Since the larval provisions are prone to fungal infestation (e.g. Strohm & Linsenmair 2001), at least some species of the Philanthini have evolved an intriguing defense mechanism that involves the PPG. Females literally embalm their prey with the secretion of the PPG (Herzner et al. 2007b, Herzner & Strohm 2007, 2008, Herzner et al. 2013, Weiss et al. 2015). This embalming reduces moisture on the prey's cuticle and hence delays fungal growth (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2011a). Since all Philanthini appear to face similar challenges regarding fungal infestation of larval provisions, their PPGs can be expected to be subject to similar natural selection pressures. Even though nothing is known about the function of the female MG, it is most likely also subject to natural, rather than sexual selection. The morphology of the PPG and MG has been shown to be rather uniform among female Philanthini (Weiss et al. 2015).

Based on the hypothesis that sexual selection causes greater interspecific diversity than natural selection (e.g. Seehausen & van Alphen 1999, Hosken & Stockley 2004, Møller & Szép 2005, Kraaijeveld et al. 2011, Wagner et al. 2012), we predict that the morphology of head glands varies more among male than among female Philanthini. Other evolutionary processes like genetic drift and mutations should affect the glands of both sexes in the same way. Since detailed morphological studies on male head glands were only available for two species of the subfamily Philanthinae, *P. triangulum* (MG: Goettler & Strohm 2008, PPG: Herzner et al. 2007a) and *Cerceris rybyensis* (MG: Ågren 1977), we conducted a comparative analysis of the PPG and MG of male Philanthinae. Using histological methods and 3D-reconstructions, we investigated males of 30 species of Philanthini, covering all major phylogenetic lineages. Moreover, we included three species of the closely related tribes Cercerini and Aphilanthopsini. Based on 14 morphological characters, comprising incidence, location, size, shape and structure of gland reservoirs, as well as histological characteristics of associated secretory cells, we performed a multivariate statistical analysis of PPGs and MGs to assess the pattern of interspecific variation in gland morphology. In order to reveal possible phylogenetic trends, we mapped gland morphology on a recent molecular phylogeny of the Philanthinae (Kaltenpoth et al. 2014). To explore the evolutionary origin and fate of important characters, we conducted ancestral state reconstruction analyses (Pagel 1999). We

discuss the interspecific variation in male head gland morphology and assess the role of sexual selection in the evolution of these glands in male Philanthinae. Using the variation of female head glands (Weiss et al. 2015) as a reference under natural selection, we test whether head gland morphology shows higher diversity in males.

## 5.3 Methods

### 5.3.1 Study material

Overall, males of 33 species and one subspecies from five genera, representing the three tribes of the crabronid subfamily Philanthinae were examined (Table 5.1). We refer to the phylogeny and phylogeography of the Philanthinae according to Kaltenpoth et al. (2014). Designation of zoogeographic regions follows Holt et al. (2013). Our main focus was on the tribe Philanthini, the so-called beewolves. The Philanthini can be grouped into five clades, largely coinciding with their geographic distribution (Kaltenpoth et al. 2014) and we investigated representatives of all of these clades (Table 5.1): One species of the basal genus *Philanthinus*, two species of a small clade of Palearctic, Indian, and Afrotropical species of the genus *Philanthus*, forming the sister group to all other *Philanthus*, ten species of a clade comprising all other Palearctic, Indian, and Afrotropical *Philanthus*, fourteen Nearctic *Philanthus* species, and three species of the Neotropical subgenus *Trachypus*. The total number of described species is four for *Philanthinus,* 136 for *Philanthus* and 31 for *Trachypus* (Pulawski 2016a, 2016b). Moreover, we included three species of the two other tribes of the Philanthinae, namely one Nearctic *Clypeadon* species (tribe Aphilanthopsini, 13 described species) and two Palearctic *Cerceris* (tribe Cercerini, 905 described species). Each species under study is assigned an ID number (Table 5.1) that is used throughout the manuscript and Supplementary Material.

**Table 5.1** Species included in the comparative morphological study of head glands of male Philanthinae. Tribe: phylogenetic affiliation of the species, ID: identification number of the species, Species: Species name, N: number of specimens examined, Country: collection site of the species, 3D: 3D-reconstruction for this species conducted (yes) or not (no).

| Tribe | ID | Species | N | Country | 3D |
|---|---|---|---|---|---|
| **Cercerini** | **1** | *Cerceris quinquefasciata* | 2 | Germany | yes |
| | **2** | *Cerceris rybyensis* | 2 | Germany | yes |
| **Aphilanthopsini** | **3** | *Clypeadon laticinctus* | 5 | USA | yes |
| **Philanthini** | **4** | *Philanthinus quattuordecimpunctatus* | 3 | Turkey | yes |
| | **5** | *Philanthus* cf. *basalis* | 1 | India | no |
| | **6** | *Philanthus pulcherrimus* | 1 | India | yes |
| | **7** | *Philanthus spec* (India) | 1 | India | yes |
| | **8** | *Philanthus venustus* | 2 | Turkey | yes |
| | **9** | *Philanthus capensis* | 1 | South Africa | yes |
| | **10** | *Philanthus coronatus* | 2 | Germany | yes |
| | **11** | *Philanthus fuscipennis* | 1 | South Africa | yes |
| | **12** | *Philanthus histrio* | 2 | South Africa | no |
| | **13** | *Philanthus loefflingi* | 3 | South Africa | yes |
| | **14** | *Philanthus melanderi* | 1 | South Africa | yes |
| | **15** | *Philanthus rugosus* | 3 | South Africa | yes |
| | **16** | *Philanthus triangulum triangulum* | 3 | Germany | no |
| | **17** | *Philanthus triangulum diadema* | 3 | South Africa | yes |
| | **18** | *Philanthus albopilosus* | 2 | USA | yes |
| | **19** | *Philanthus barbiger* | 3 | USA | yes |
| | **20** | *Philanthus bicinctus* | 2 | USA | yes |
| | **21** | *Philanthus crotoniphilus* | 2 | USA | yes |
| | **22** | *Philanthus gibbosus* | 3 | USA | no |
| | **23** | *Philanthus gloriosus* | 2 | USA | yes |
| | **24** | *Philanthus multimaculatus* | 2 | USA | yes |
| | **25** | *Philanthus occidentalis* | 2 | USA | no |
| | **26** | *Philanthus pacificus* | 1 | USA | yes |
| | **27** | *Philanthus parkeri* | 1 | USA | yes |
| | **28** | *Philanthus politus* | 2 | USA | yes |
| | **29** | *Philanthus psyche* | 1 | USA | yes |
| | **30** | *Philanthus pulcher* | 1 | USA | yes |
| | **31** | *Philanthus ventilabris* | 1 | USA | yes |
| | **32** | *Trachypus elongatus* | 2 | Brazil | yes |
| | **33** | *Trachypus flavidus* | 2 | Brazil | no |
| | **34** | *Trachypus patagonensis* | 1 | Brazil | no |

### 5.3.2 Histology

Wasps were caught in the field in their territories or at flowers. They were cold anesthetized, decapitated and heads were fixed either in formalin-ethanol-acetic acid, alcoholic Bouin, or, in four cases, 100% ethanol (Adam & Czihak 1964). After fixation, heads were rinsed, dehydrated in a graded ethanol series and propylene oxide, and embedded in Epon 812 (Polysciences Europe GmbH, Eppelheim, Germany). To facilitate the infiltration of the embedding medium into large heads, lateral parts of both compound eyes were cut off after fixation. Continuous series of

sagittal semithin sections (4 µm) were cut with a microtome (Reichert Ultracut; Leica Microsystems AG, Wetzlar, Germany) equipped with a diamond knife and a large trough, mounted on microscope slides, and stained with toluidine blue (Adam & Czihak 1964). The resulting series of histological sections were investigated by light microscopy (bright field, differential interference contrast, and phase contrast; Zeiss Axiophot 2; Carl Zeiss Microscopy GmbH, Oberkochen, Germany; Leica DMLS, Leica GmbH, Wetzlar, Germany).

Designation of glands was done according to the site of their openings. Reservoirs opening near the base of the mandibles were regarded as MGs and reservoirs opening to the pharynx just proximal to the hypopharyngeal plate were regarded as PPGs. Secretory cells associated with the gland reservoirs were classified according to Noirot and Quennedey (1974) whenever possible; such cells will be referred to as 'NQ-class cells'. In addition, we detected presumably secretory cells not matching the classification of Noirot and Quennedey (1974). We include these cells as morphological characters in our analysis (see 5.3.4.1) but will provide extensive histological and ultrastructural details elsewhere.

All species also possessed a hypopharyngeal gland. We did not include this gland in our analysis, because several aspects contradict a role in territory marking: (1) the gland seems to be involved in nutrition and digestion (Cruz-L&im & Costa 1998, do Amaral & Caetano 2005, Billen et al. 2013), (2) it does not have a reservoir, and (3) using gas chromatography and mass spectrometry, we did not find volatile components in this gland (Strohm et al., *unpublished data*).

### 5.3.3 3D-Reconstruction

To visualize the overall morphology of head glands and to facilitate comparison among species, 3D-reconstructions of the head glands were generated for 27 of the 34 investigated taxa (Table 5.1). For two *Trachypus* and five *Philanthus* species no complete series of sections were available (Table 5.1); however, also for these species the available histological sections were sufficient to allow for the determination of most gland characters (see 5.3.4.1). Due to deficient quality of a part of the sections, reconstruction was only possible for one side of the head for *Philanthus capensis* (ID 9)*, Philanthus gloriosus* (ID 23), and *Philanthus multimaculatus* (ID 24). For 3D-reconstruction, continuous series of semithin sections of one individual per species (on average 560 sections per head; 14,980 sections in total) were photographed using a digital microscope camera (Olympus DP20; Olympus, Hamburg, Germany) attached to a light microscope (Zeiss Axiophot 2) using 2.5x or 5x PlanNeofluar objectives. The digital images were automatically

aligned to each other using the software TrakEM2 (Cardona et al. 2012) for the image processing software Fiji (Schindelin et al. 2012); all alignments were checked and manually corrected if necessary. The outer margin of the epithelium surrounding the reservoirs of the MG and the PPG as well as the pharynx were then marked as 3D-objects in TrakEM2 by manually outlining them in each picture of a series. For *Philanthus rugosus* (ID 15), additionally secretory cells of the MG and the PPG as well as the brain and the ocelli were marked. Finally, 3D-reconstructions were calculated and visualized using Fiji's 3D-viewer plug-in (Schmid et al. 2010).

### 5.3.4 Statistical analysis of gland morphology

#### *5.3.4.1 Morphological characters*

Based on an extensive examination of both semithin histological sections and 3D-reconstructions, we defined 14 morphological characters of the PPG and MG for a comparative statistical analysis of the head glands of male Philanthinae. These characters comprise information on the incidence, relative size, structure and overall shape of the glands, their location within the head capsule, as well as the type and arrangement of associated gland cells. Character states were categorized and numerically coded for statistical analysis. Due to partial deficiencies in the histological sections not all character states could be determined for all species. Detailed descriptions of the characters and character states are given in Supplementary Material section 5.6.1. In brief, the defined characters were: (1) 'Overall structure of the PPG', (2) 'Size of the PPG relative to the head capsule', (3) 'Modifications of PPG morphology', (4) 'Branching of the PPG', (5) 'Numbers of openings of the lower part of the PPG to the pharynx', (6) 'Structure of the inner walls of the PPG', (7) 'Type of gland cells associated with the PPG', (8) 'Presence of the MG', (9) 'Overall structure of the MG', (10) 'Size of the MG relative to the head capsule', (11) 'Location of the MG in the head capsule', (12) 'Branching of the MG', (13) 'Structure of the inner walls of the MG', (14) 'Type of gland cells associated with the MG'. While the volume of a gland may vary due to differences in filling status, the longitudinal extension within the head capsule that we used as a measure of gland size is only slightly affected. If several specimens were available for a species, these had very similar morphology and did not differ with regard to the character states.

#### *5.3.4.2 Data matrix for statistical analysis*

The pronounced variation among species (see Results) required the differentiation of many character states. Since only a limited number of species could be analyzed there was only a low

number of cases for some character states (see 5.6.1 and Table S5.1, Supplementary Material). Therefore, in addition to a dataset comprising all differences observed among species ('full dataset', Table S5.1), we created a second dataset, in which we pooled character states wherever reasonable ('combined dataset', Table S5.2, Supplementary Material section 5.6.1) and that we used for statistical analyses.

*5.3.4.3 Categorical principal components analysis*

To reveal patterns of character distribution among species, a categorical principal component analysis (CATPCA) was conducted using the program 'CATPCA' (Meulman et al. 2004a) implemented in the SPSS Categories module (SPSS version 21.0, IBM; Chicago, IL, USA). Two species were excluded from the CATPCA: For the Neotropical *Trachypus patagonensis* (ID 34) the insufficient quality of the single available series of histological sections only allowed to obtain reliable data on MG but not on PPG morphology (Table S5.1). Moreover, males of the Nearctic *Philanthus albopilosus* (ID 18) lacked well-developed head glands (see Results). Hence, the large difference of *P. albopilosus* to the other philanthine species would have unnecessarily lowered the quality parameters of the CATPCA solution. More details on the implementation of the CATPCA are given in Supplementary Material section 5.6.2.1.

To test whether there was an opposing trend between MG and PPG with regard to their size and complexity, we conducted phylogenetic generalized least squares regressions based on the molecular phylogeny of Kaltenpoth et al. (2014). As with the CATPCA, *P. albopilosus* was excluded from this analysis, as well as the Nearctic *Philanthus gibbosus* (ID 22), for which the size of the PPG reservoir could not be assessed (Tables S5.1 and S5.2). Moreover, since the molecular phylogeny comprised only one unidentified *Cerceris* species (Kaltenpoth et al. 2014), we included only *C. rybyensis* (ID 2; omitting *Cerceris quinquefasciata*, ID 4). We used the package 'ape' (Paradis et al. 2004) in R (Version 3.3.3) (R Core Team 2017) to test for a correlation between MG and PPG size and MG and PPG complexity with correction for phylogenetic relationships (for more details see Supplementary Material section 5.6.2.2).

*5.3.4.4 Hierarchical cluster analysis and phylogenetic trends in gland morphology*

We tested for phylogenetic trends in gland morphology using a cophylogenetic analysis between a morphology-based dendrogram resulting from a hierarchical cluster analysis (HCA) and a molecular phylogeny (Kaltenpoth et al. 2014). The HCA was based on 13 of the 14 gland

characters (see Supplementary Material section 5.6.2.3) and was conducted in PAST (Version 2.08b) (Hammer et al. 2001) with the Bray-Curtis-index as a measure of dissimilarity and 'unweighted pair-group averages' as clustering algorithm; the number of bootstrap replicates was set to 10,000. Cophylogenetic analyses are mostly employed to test for coevolution of parasites and their hosts. Treating the morphology-based dendrogram as 'parasite tree' and the molecular phylogeny of the Philanthinae (Kaltenpoth et al. 2014) as 'host tree', the congruence between the two was tested for statistical significance using the software tool Jane 4 (Conow et al. 2010). Details on the implementation of the HCA and the cophylogenetic analysis are given in Supplementary Material sections 5.6.2.3 and 5.6.2.4.

*5.3.4.5 Ancestral state reconstructions*

Our investigations revealed that two major aspects of the head glands of male Philanthinae, the MG as well as the presumed secretory cells of the PPG, showed a complex phylogenetic distribution including losses and regains (see Results). Based on the molecular phylogeny (Kaltenpoth et al. 2014), we conducted ancestral state reconstructions (ASR) (Pagel 1999) for the presence of both the MG (character 8, Tables S5.1 and S5.2, Supplementary Material section 5.6.1) and the secretory cells of the PPG (state 0 *vs.* all other states of character 7, Tables S5.1 and S5.2) using the software tool Mesquite (Version 3.04) (Maddison & Maddison 2015). As above, since the molecular phylogeny comprised only one unidentified *Cerceris* species, we conducted the ASR with only *C. rybyensis* (ID 2) and *Clypeadon laticinctus* (ID 3) as outgroup species (omitting *Cerceris quinquefasciata*, ID 4). We applied maximum likelihood (ML) approaches using asymmetrical Markov k-state 2 parameter models with the rate of change between the two character states (i.e. absence *vs.* presence of the MG and the secretory cells of the PPG, respectively) set to 1. Since for both traits the likelihood of gain *vs.* loss is not known, we tested different bias ratios for gains *vs.* losses ranging from 10 (i.e. gains ten times more frequent than losses) to 0.1 (i.e. losses ten times more frequent than gains).

*5.3.4.6 Comparison of morphological diversity in males and females*

To formally evaluate the hypothesis that the diversity among males is larger than among females, we compiled an aggregated matrix of gland characters of males of 32 species and females of 28 species (data for females taken from Weiss et al. 2015; see Table S2.2 in section 2.6.2 of chapter 2). Most characters are shared by both sexes and the respective character states could be simply combined. However, some characters or character states had to be recoded because they were

assessed differently in the sexes or the character states were more finely differentiated in females. Based on the aggregated matrix, we conducted a CATPCA as described above to illustrate the distribution of males and females with regard to their gland morphology. To test for a difference in diversity between males and females, we calculated Shannon diversity indices among the character states of the characters that occur in both sexes (four characters that are restricted to either males or females had to be omitted) and compared these values using an exact Wilcoxon matched pair test. For more details see Supplementary Material section 5.6.7.

## 5.4 Results

### 5.4.1 General aspects of gland morphology

In all species of Philanthinae under study, males possess either an MG, or a PPG, or both and, with one exception (*P. albopilosus*, ID 18), at least one of these glands occupies a considerable part of the head capsule. Nineteen of the 33 investigated species possess an MG that is located in the front part of the head capsule anterior to the brain and, depending on its size, may extend behind the brain, lateral from or subjacent to the PPG. The MG comprises paired reservoirs opening at the dorsal side of the mandible base and extending laterally and dorsally on both sides of the head capsule, in some cases even reaching behind the brain (Figure 5.1, *P. rugosus*). Some species have only a lower MG reservoir opening at the ventral side of the mandible base and extending backwards. A few species possess both parts. MG reservoirs are surrounded by a monolayered epithelium that is moderately thick in most species. However, in some species with only an upper MG, the epithelium is distinctly thinner. The epithelial cells bear an apical cuticular intima that regularly forms a variety of conspicuous structures. Moreover, there is interspecific variation with regard to the types of secretory cells associated with the MG (see below).

All 33 investigated species possess a PPG, clearly identified by its connection to the pharynx anterior to the brain and posterior to the hypopharyngeal plate (Figure 5.1). The PPG also shows considerable interspecific variation. In most species, the main upper part of the PPG basically consists of two pairs of lateral evaginations: one pair extending dorsally and in some species even around the brain (dPPG in Figure 5.1) and a second pair located anterior to the brain and extending laterally towards the ventral rims of the compound eyes (aPPG in Figure 5.1, for the delineation of the two parts see also Figure S5.1 A and B, Supplementary Material section 5.6.1). The anterior part may reach the compound eyes and, in some species, the base of the mandibles.

In 14 of the 33 investigated species, there is an additional, smaller, lower part of the PPG consisting of an unpaired ventral evagination of the pharynx (Figure S5.1 F). The walls of all parts of the PPG consist of a (partly very thin) monolayered epithelium with an apical cuticular intima. The epithelial cells generally bear hairs or scales that extend into the lumen of the gland.

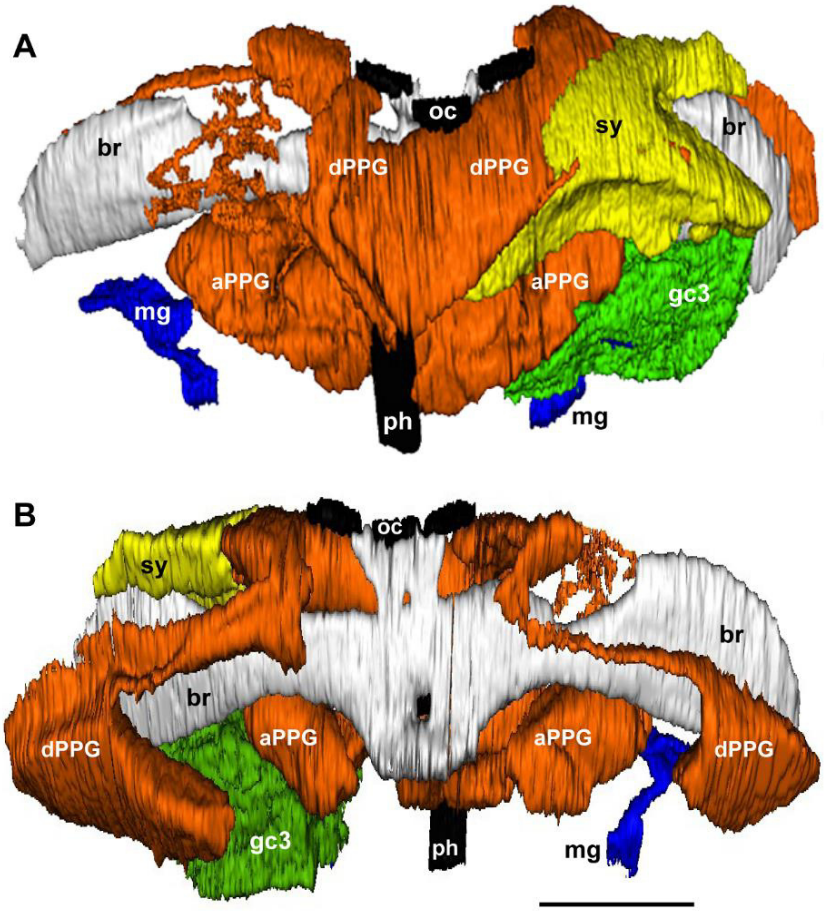


**Figure 5.1** 3D-reconstruction of the internal structures of a male *Philanthus rugosus* head. **(A)** Anterior view, **(B)** posterior view. The upper postpharyngeal gland reservoir (PPG; orange) originates dorsally from the pharynx (black) and basically consists of two pairs of lateral evaginations, one extending dorsally around the brain (light grey) (dPPG; see also Figure S5.1 A) and one extending laterally anterior to the brain (aPPG; see also Figure S5.1 B). The fine branches originating from the dorsal part of the upper PPG (see also Figure S5.1 C) are surrounded by syncytia of secretory cells (yellow, shown only for the left side of the head). The upper mandibular gland reservoirs (MG; blue) have their openings at the dorsal mandibular base and extend laterally. The MG is associated with single NQ-class 3 gland cells (green, shown only for the left side of the head capsule). Abbreviations: aPPG, anterior parts of the upper PPG reservoir; br, brain; dPPG, dorsal parts of the upper PPG reservoir; gc3, single NQ-class 3 gland cells associated with the MG; mg, upper MG reservoir; oc, ocelli; ph, pharynx; sy, syncytia of secretory cells associated with the fine branches of the dorsal part of the upper PPG. Scale bar = 0.5 mm.

The reservoirs of both glands may be associated with different types of cells (Figure 5.2 and Figure S5.2, Supplementary Material section 5.6.4.1; see also Figure 5.1 for the location of the cells). These cells presumably have secretory functions given their close proximity or direct contact to the reservoirs and the abundance of vesicles and nucleoli (Figure 5.2). The gland cells of the MG can be differentiated into three types. In some species there are typical NQ-class 3 cells (Noirot & Quennedey 1974), i.e. complexes of a secretory cell and a canal cell, the latter forming conspicuous end apparatus and canals that connect the secretory cell to the lumen of the MG (Figure 5.2 A). In other species, several NQ-class 3 cells are aggregated in acini (Figure 5.2 B). The third type comprises secretory cells that are located directly at the wall of the reservoir and bear end apparatus but no canals (Figure 5.2 C). Though these cells appear to be complexes of two cells, thus resembling NQ-class 3 cells, we assign them to a different character state to account for the lack of visible canals (see also Supplementary Material section 5.6.1).

The cells associated with the PPG can occur either as aggregations of mononuclear cells (superficially resembling the acini of the MG) (Figure 5.2 D) or as multinuclear syncytia (Figure 5.2 E) (see below and Supplementary Material section 5.6.1), both showing clear signs of secretory activity: large nuclei, conspicuous nucleoli and numerous vesicles (black arrowheads in Figure 5.2 E). However, these cells are clearly not NQ-class 3 cells, since they lack an end apparatus and canals. Moreover, they are not part of the gland epithelium and are, thus, not NQ-class 1 cells either. Remarkably, the PPG reservoir itself is extensively ramified with the thinnest branches reaching into the cell aggregations or syncytia (black arrow and inset in Figure 5.2 E). In some species the cell aggregations or syncytia are interspersed with conspicuous small rounded cells with barely any cytoplasm (white arrows in Figure 5.2 D and F).

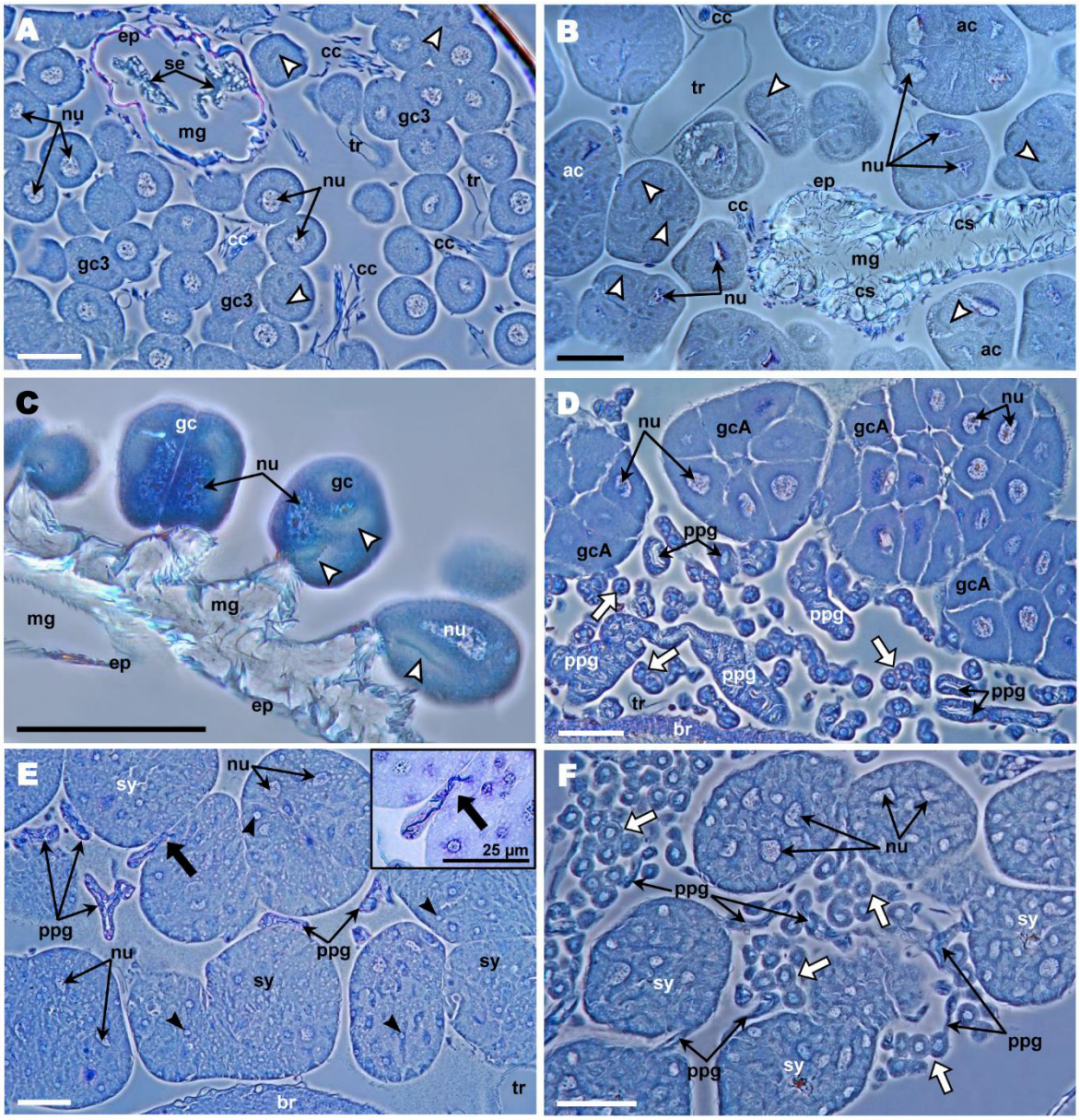


**Figure 5.2** Semithin sagittal sections through the heads of male Philanthinae. **(A)** Single NQ-class 3 gland cells, i.e. complexes of a secretory cell and a canal cell, the latter forming a conspicuous end apparatus (white arrow heads) and canal that connects the secretory cell to the lumen of the MG (*Philanthus multimaculatus*, ID 24); **(B)** Acini of NQ-class 3 gland cells with end apparatuses (white arrow heads) connected to the MG reservoir by bundles of conducting canals (*Philanthus t. diadema*, ID 17); **(C)** Single gland cells possessing end apparatus (white arrow heads), thus resembling NQ-class 3 cells, but directly associated with the wall of the MG reservoir without canals (*Clypeadon laticinctus*, ID 3); **(D)** Aggregations of mononuclear secretory cells surrounding the fine branches of the PPG reservoir, interspersed with small rounded cells (white arrows) (*Philanthus venustus*, ID 8); **(E)** Multinuclear syncytia of secretory cells, containing many vesicles (black arrow heads), and in close contact to the fine branches of the PPG reservoir (thick black arrow; inset: detail of a PPG branch terminating in syncytium) (*Philanthus histrio*, ID 12); **(F)** Multinuclear syncytia of secretory cells surrounding the fine branches of the PPG reservoir and interspersed with small cells (white arrows) (*Philanthus crotoniphilus*, ID 21). Abbreviations: ac, acini of NQ-class 3 cells; br, brain; cc, conducting canal; cs. cuticular spines; ep, epithelium of the MG; gc. secretory cells not resembling NQ-class cells; gcA, aggregations of mononuclear secretory cells; gc3, NQ-class 3 gland cells; mg, lumen of the mandibular gland; nu, nucleus with nucleoli; ppg, fine branches of the postpharyngeal gland; se, secretion within the MG; sy, multinuclear syncytia; tr, tracheole. Scale bars [except inset in (E)] = 50 µm.

### 5.4.2 Pattern of interspecific variation in gland morphology

Both PPG and MG show remarkable interspecific variation with respect to their incidence, size and shape (Figure 5.3), as well as the fine structure of the gland reservoirs and the type and arrangement of the associated secretory cells (Figure 5.2). Character states for the species under study are given in Tables S5.1 and S5.2, Supplementary Material section 5.6.1. The CATPCA analysis based on eleven morphological characters (Table S5.2) sorted the species under study into three well defined groups (I-III, see below) and two species largely separated from these groups (Figure 5.4). The first two dimensions of the CATPCA together explained 94% (63% and 31%, respectively) of the variance in the dataset and were supported by a total Cronbach's α of 0.99 (maximum value = 1), indicating the high reliability of the detected pattern in the dataset (Heiser & Meulman 1994). Size and complexity of MG and PPG strongly contribute to the separation of the groups, and their vectors point in opposite directions. Yet, according to phylogenetic independent regression analyses there was no significant correlation between size ($N = 30$, $r = -0.47$, $p = 0.136$) or complexity ($N= 30$, $r = -0.6$, $p = 0.14$) of MGs and PPGs across species. However, due to the comparatively small set of species in our analysis (Münkemüller et al. 2012) this result bears some uncertainty.

#### *5.4.2.1 Group I: Species possessing large MGs but only small PPGs*

The first group of species as assigned by the CATPCA (Figure 5.4) is characterized by complex and large MGs, but only small and simple PPGs. In all species of this group, the MG reservoir (turquoise in Figure 5.3) opens at the dorsal side of the mandible base and is bordered by a rather thin monolayered epithelium in direct contact with gland cells that show the typical end apparatus of NQ-class 3 cells, but no canals (Figure 5.2 C). *Cerceris rybyensis* (ID 2) additionally possesses a second reservoir (dark blue in Figure 5.3) with a distinctly thicker, yet likewise monolayered epithelium and being exceptional in having two openings, one dorsally and one ventrally at the mandibular base. This additional reservoir is associated with typical NQ-class 3 cells with end apparatus and canals. The small PPG reservoirs of group I species are not associated with any cells that show signs of secretory activity. Notably, group I solely comprises the three investigated species of the tribes Cercerini and Aphilanthopsini (IDs 1-3).

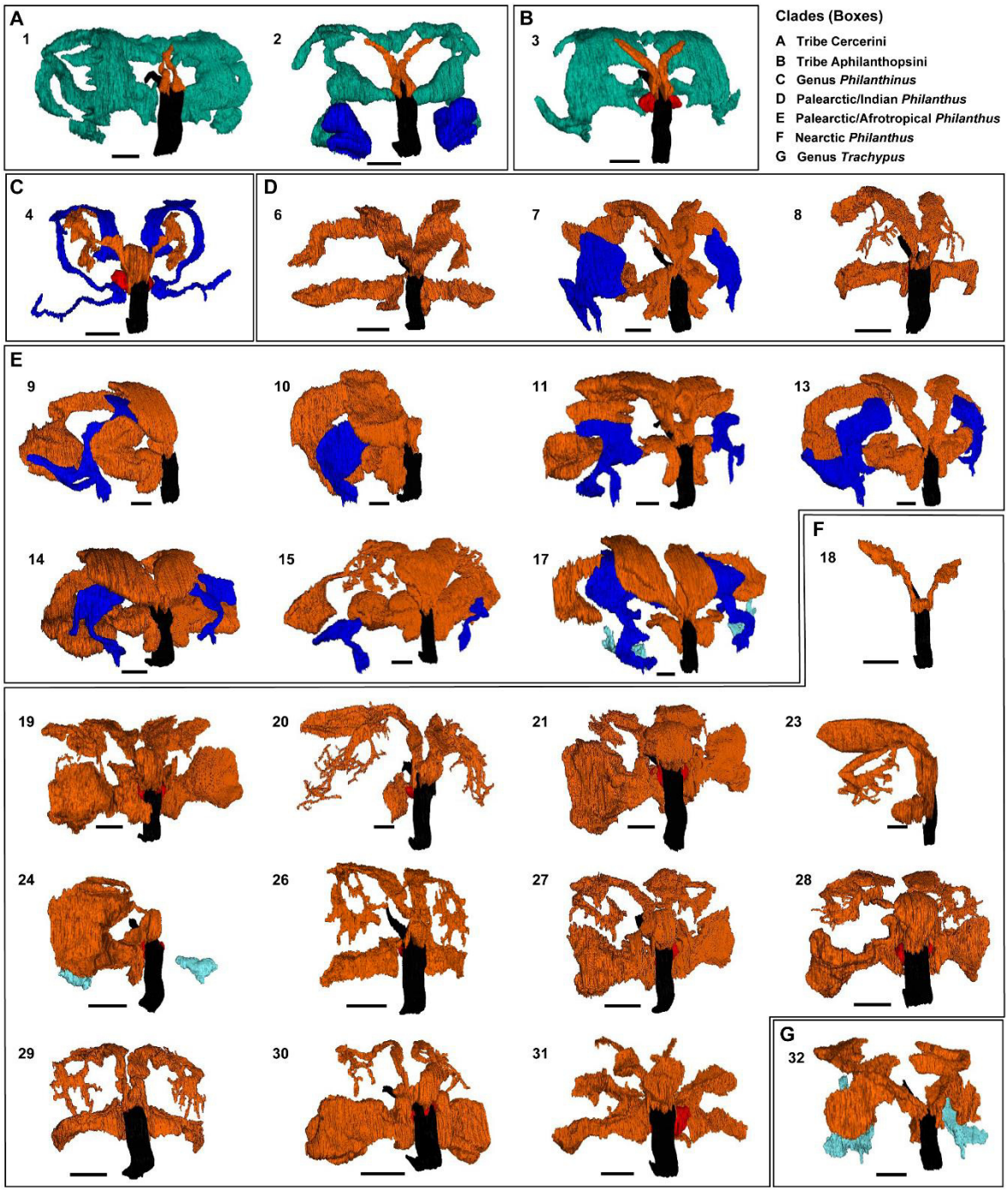


**Figure 5.3** 3D-reconstructions of the postpharyngeal gland (PPG) and the mandibular gland (MG) of male Philanthinae. Species IDs (corresponding to Table 5.1): (1) Cerceris quinquefasciata, (2*) Cerceris rybyensis*, (3) *Clypeadon laticinctus*, (4) *Philanthinus quattuordecimpunctatus*, (6) *Philanthus pulcherrimus*, (7) *Philanthus spec.* (India), (8) *Philanthus venustus*, (9) *Philanthus capensis*, (10) *Philanthus coronatus*, (11) *Philanthus fuscipennis*, (13) *Philanthus loefflingi*, (14) *Philanthus melanderi*, (15) *Philanthus rugosus*, (17) *Philanthus triangulum diadema*, (18) *Philanthus albopilosus*, (19) *Philanthus barbiger*, (20) *Philanthus bicinctus*, (21) *Philanthus crotoniphilus*, (23) *Philanthus gloriosus*, (24) *Philanthus multimaculatus*, (26) *Philanthus pacificus*, (27) *Philanthus parkeri*, (28) *Philanthus politus*, (29) *Philanthus psyche*, (30) *Philanthus pulcher*, (31) *Philanthus ventilabris*, (32*) Trachypus elongatus*. Boxes indicate phylogeographic classification of species (according to Kaltenpoth et al. 2014), see key in figure. Color code for 3D-structures: orange, upper part of the PPG; red, lower part of the PPG; dark blue, upper part of the MG; light blue, lower part of the MG, turquoise, thin-walled MG reservoir of the Cercerini and Aphilanthopsini; black, pharynx. Due to limited availability of serial histological sections, for species (9), (10), and (23) only the right side of the paired gland reservoirs could be reconstructed, while for species (24), both reservoirs of the MG, but only the right half of the PPG are depicted; for species (6), (7), (10) - (15), and (31), the fine branches originating from the main PPG reservoir [see e.g. species (8) and (20)] could not be reconstructed based on semithin section due to their very fine structure and high number. Scale bars = 0.25 mm.

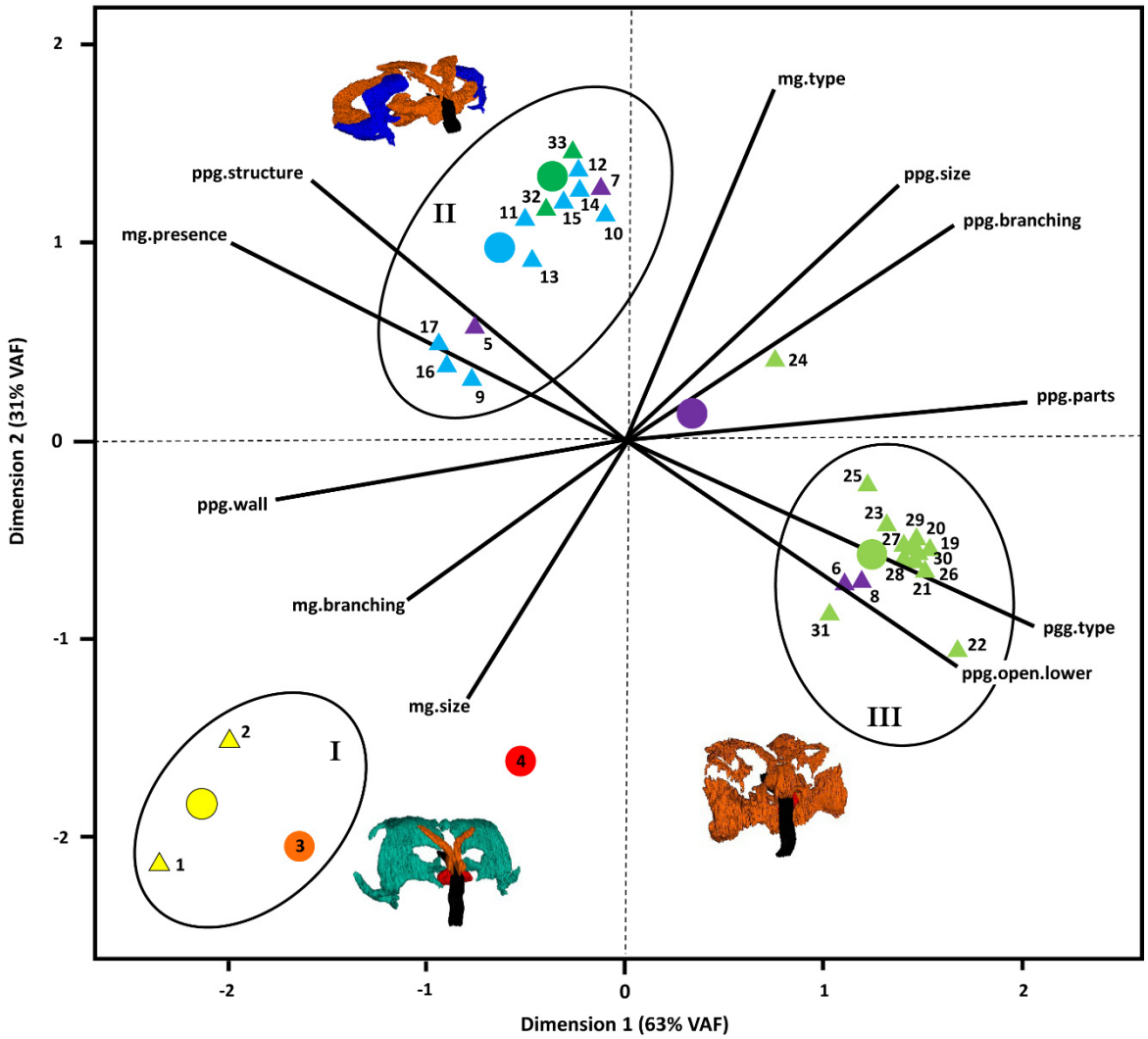


**Figure 5.4** First two dimensions (VAF: percent of variance accounted for) of the CATPCA of the head gland morphology of male Philanthinae. Based on the morphology of their head glands, the species form three distinct groups (Ellipses; exemplary 3D-reconstructions: (I) *Cerceris quinquefasciata*, (II) *Philanthus rugosus*, (III) *Philanthus politus*). Triangles: object scores of single species (IDs correspond to Table 5.1), Vectors: component loadings of morphological characters, Circles: Group centroids of the different phylogenetic and phylogeographic clades (according to Kaltenpoth et al. 2014) included as a supplementary variable. Note that for each of the two genera *Clypeadon* (orange) and *Philanthinus* (red) only one species was included in the analysis, thus, their object scores are identical to their group centroids. Color code: yellow, genus *Cerceris*; orange, genus *Clypeadon*; red, genus *Philanthinus*; purple, Palearctic/Asian *Philanthus*; blue, Palearctic/Afrotropical *Philanthus*; light green, Nearctic *Philanthus*; dark green, genus *Trachypus*. Abbreviations of morphological characters (numbering corresponds to section 5.3.4.1): ppg.structure, (1) overall structure of the PPG; ppg.size, (2) size of the PPG relative to the head capsule; ppg.parts, (3) modifications of PPG morphology; ppg.branching, (4) branching of the PPG; ppg.open.lower, (5) numbers of openings of the lower part of the PPG to the pharynx; ppg.wall, (6) structure of the inner walls of the PPG; ppg.type, (7) type of gland cells associated with the PPG; mg.presence, (8) presence of the MG; mg.size, (10) size of the MG relative to the head capsule; mg.branching, (12) branching of the MG; mg.type, (14) type of gland cells associated with the MG.

### *5.4.2.2 Group II: Species possessing both well-developed MGs and PPGs*

The second group comprises 12 species (including the two subspecies of *P. triangulum*, IDs 16 and 17) (Figure 5.4) that possess both large, complex PPGs and mostly medium-sized, yet well-developed MGs with fairly thick epithelia. Most members of this group possess only the upper

part of the MG (dark blue in Figure 5.3), whereas *Philanthus* cf. *basalis* (ID 5), *P. t. triangulum* (ID 16), and *P. t. diadema* (ID 17) possess both upper and lower parts and *Trachypus elongatus* (ID 32) possesses only the lower part of the MG (light blue in Figure 5.3). In nine species of group II, the MG is associated with acini made up of NQ-class 3 cells with canals jointly connecting an acinus with the reservoir (Figure 5.2 B). Yet, the closely related *Philanthus histrio* (ID 12) and *P. rugosus* (ID 15) as well as the two *Trachypus* species (IDs 32 and 33) possess single NQ-class 3 cells (Figure 5.2 A).

In eight species of group II, the PPG reservoir is extensively ramified and associated with cells that show clear signs of secretory activity. In seven of these species the cells at the PPG are syncytia (Figure 5.2 E); only in *Trachypus flavidus* (ID 33) these cells are aggregations of mononuclear cells. The remaining five species of group II, *P.* cf. *basalis* (ID 5), *P. capensis* (ID 9), *P. t. triangulum* (ID 16), *P. t. diadema* (ID 17), and *T. elongatus* (ID 32) possess large, un-ramified more or less tube-shaped PPGs and neither the cells of the PPG epithelium nor surrounding cells show signs of secretory capacity. Group II comprises all but two of the investigated Palearctic, Indian, and Afrotropical species of the genus *Philanthus* (IDs 5, 7 and 9-17), as well as the two Neotropical species *T. elongatus* (ID 32) and *T. flavidus* (ID 33).

*Trachypus patagonensis* (ID 34) that was not included in the CATPCA (see 5.3.4.2) would probably also be placed in this group. Its MG consists of both upper and lower part associated with single NQ-class 3 cells and its PPG is tubular and not associated with secretory cells.

*5.4.2.3 Group III: Species with large, complex PPGs but no MGs*

The third group is rather narrowly defined and comprises 14 *Philanthus* species characterized by completely lacking an MG but possessing large and extensively ramified PPGs (Figure 5.3) associated with secretory cells. *Philanthus venustus* (ID 8) deviates from the other members of group III in that the secretory cells of its PPG are not syncytia but aggregations of mononuclear cells (Figure 5.2 D), similar to *T. flavidus* (ID 33) in group II. Only in species of group III are the syncytia or cell aggregations associated with the PPG branches interspersed with small rounded cells with barely any cytoplasm (white arrows in Figure 5.2 D and F). Most species of group III have a Nearctic distribution, the exceptions being the Indian *Philanthus pulcherrimus* (ID 6) and the Palearctic *Philanthus venustus* (ID 8).

*5.4.2.4 Divergent species*

Two species included in the CATPCA are separated from the three main groups. One is *P. multimaculatus* (ID 24), the only Nearctic species in our dataset whose males have an MG. Like the Neotropical *T. elongatus* (ID 32) it has only the lower part of the MG (Figure 5.3). In the CATPCA it is located between its MG-less Nearctic relatives of group III and the Afrotropical, Palearctic and Neotropical species of group II that all possess MGs. The second separated species is *P. quattuordecimpunctatus* (ID 4), whose males have a well-developed tube-shaped MG, associated with cells akin to NQ-class 3 gland cells that, however, lack conducting canals, resembling group I in this respect. The upper part of their PPG extends backwards around the brain, like in the species of group II, and is not associated with any secretory cells. Moreover, the PPG of *P. quattuordecimpunctatus* is unique among all investigated species in that its reservoir consists of a complex network of lamellae (not shown) as opposed to the tubular ramifications of the other species.

*Philanthus albopilosus* (ID 18; not in CATPCA, see 5.3.4.3 and Discussion) stands out from all other species. Its males not only completely lack an MG, like most of their Nearctic congeners, but also have a largely reduced PPG that consists of only small evaginations of the pharynx (Figure 5.3) without any secretory cells, similar to the PPGs of group I.

**5.4.3 Phylogenetic trend in gland morphology**

As summarized in Figure 5.5, the gland morphology of male Philanthinae partly coincided with phylogenetic groups, but there is also considerable diversity within clades and several species deviate from their closest relatives. To test whether there is an overall phylogenetic trend in gland morphology we conducted a HCA (Figure S5.3, Supplementary Material section 5.6.4.2) based on the morphological characters of MG and PPG and compared the resulting dendrogram with the molecular phylogeny of the Philanthinae (Kaltenpoth et al. 2014). The HCA largely corroborated the pattern found in the CATPCA (for details on the clustering of species see Supplementary Material section 5.6.4.2). Notably, the HCA dendrogram shows a highly significant congruency with the molecular phylogeny (cophylogenetic analysis, all tested parameter combinations: $p < 0.001$).

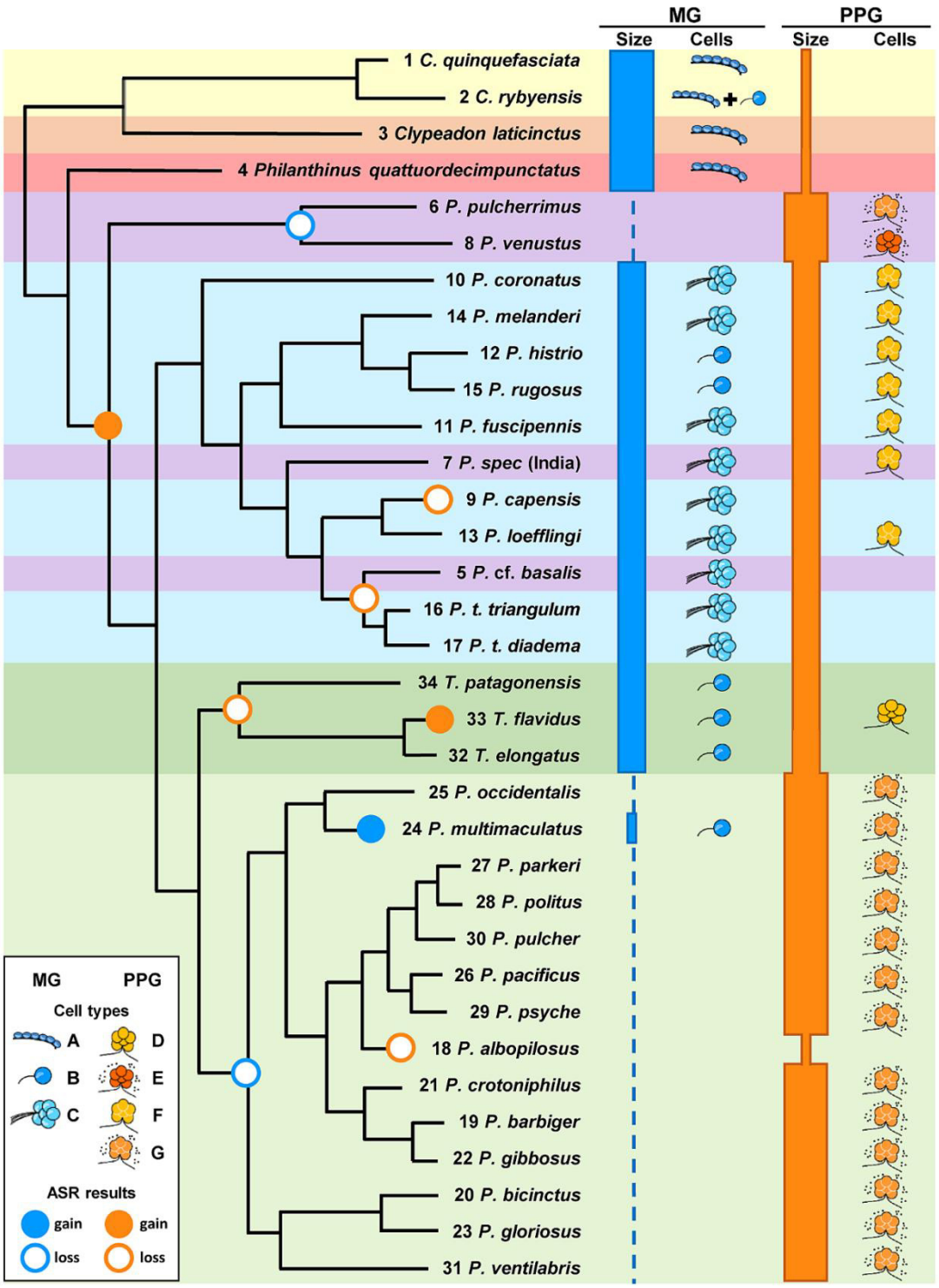


**Figure 5.5** Summary of the phylogenetic trends and deviations in size of the MG and PPG (bar thickness indicates relative size, dotted line indicates absence) and type of associated gland cells ('Cells') among male Philanthinae. Circles at nodes indicate likely events of gain or loss of the MG or the PPG (symbols see key in figure; for more details see Figure S5.4 and S5.5, Supplementary Material section 5.6.4.3). Species IDs correspond to Table 5.1. Color code of phylogeographic clades (according to Kaltenpoth et al. 2014): yellow, genus *Cerceris*; orange, genus *Clypeadon*; red, genus *Philanthinus*; purple, Palearctic/Asian *Philanthus*; blue, Palearctic/Afrotropical *Philanthus*; light green, Nearctic *Philanthus*; dark green, genus *Trachypus*). Pictograms of cell types (labeling see key in figure): (A) single gland cells, showing end apparatuses but directly associated with the wall of the MG reservoir without canal cells (B) single NQ-class 3 gland cells; (C) acini of several NQ-class 3 cells with bundles of conducting canals; (D) aggregations of several gland cells directly associated with very fine branches of the PPG; (E) as in (D), but interspersed with small rounded cells; (F) syncytia of secretory cells directly associated with very fine branches of the PPG; (G) syncytia as in (E), but interspersed with small rounded cells. Dendrogram modified after the molecular phylogeny of Kaltenpoth et al. (2014).

### 5.4.4 Phylogenetic history of the MG

The MG shows a complex phylogenetic pattern of incidence among male Philanthinae (Figure 5.5). A maximum likelihood ASR using an unbiased model (bias = 1, Figure S5.4, Supplementary Material section 5.6.4.3) suggests the presence of an MG as the ancestral state of the subfamily Philanthinae as well as of the tribe Philanthini and of the genus *Philanthus* (including *Trachypus*). Accordingly, the MG would have been independently lost twice within the genus *Philanthus*, namely in the last common ancestor of the clade comprising *P. pulcherrimus* (ID 6) and *P. venustus* (ID 8) (ML probability 96%) and in the last common ancestor of the Nearctic *Philanthus* species (ML probability 100%) (Figure S5.4). In the Nearctic *P. multimaculatus* (ID 24), however, the MG must have been regained (Figure S5.4). This result did not change if losses were assumed to be more frequent than gains (bias < 1) and also if gains were assumed to be slightly more likely than losses (up to a bias of 1.5). Varying the bias further in favor of gains (bias ≥ 2.3), however, led to ambiguous or deviating results for some nodes within the genus *Philanthus* (including *Trachypus*) (Figure S5.4). Yet, even with a bias of 10 the analysis indicated the presence of an MG as the ancestral state for both the Philanthinae and the Philanthini (Figure S5.4).

### 5.4.5 Phylogenetic history of the secretory cells of the PPG

The phylogenetic pattern of the presumed secretory cells of the PPG is even more complex (Figure 5.5). According to an unbiased maximum likelihood ASR (bias = 1), the secretory cells of the PPG were most likely absent in the last common ancestors of both the Philanthinae and the Philanthini and first occurred in the last common ancestor of *Philanthus* (including *Trachypus*) (ML probability 100%; Figure 5.5, Figure S5.5, Supplementary Material section 5.6.4.3). Within *Philanthus*/*Trachypus*, the secretory cells would then have been independently lost four times (Figure 5.5, Figure S5.5), namely in the last common ancestor of the Neotropical *Trachypus* (IDs 32-34) (ML probability 71%), in the Nearctic *P. albopilosus* (ID 18), in the last common ancestor of the clade containing the two subspecies of *P. triangulum* (IDs 16 and 17) and *P.* cf. *basalis* (ID 5) (ML probability 87%) as well as in the related Paleotropical *P. capensis* (ID 9), while the closely related *Philanthus loefflingi* (ID 13) has retained the secretory cells. Hence, one species, *T. flavidus* (ID 33), must have regained the secretory cells of the PPG (Figure S5.5). Varying the bias strongly in favor of gains over losses (bias ≥ 4), resulted in a somewhat different evolutionary scenario in that the secretory cells of the PPG would have been lost in the last common ancestor of both the *P. capensis*-clade and the *P. triangulum*-clade and then regained in *P. loefflingi* (Figure S5.5).

### 5.4.6 Comparison of morphological diversity in males and females

The CATPCA based on the aggregate matrix of character states for males and females reveals a clear distinction between the sexes (Figure S5.6, Supplementary Material section 5.6.6). Whereas data points for females are largely clumped, the data points for males are much more scattered and show two main aggregations similar to the CATPCA including only males. Diversity estimates of character states among characters of gland morphology were significantly higher in males (mean ± s.d.: 1.12 ± 0.27) than in females (0.34 ± 0.32; Wilcoxon matched pairs test: N = 9 characters, exact p = 0.004).

## 5.5 Discussion

There are several comparative phylogenetic studies on secondary sexual traits (e.g. Kopp & True 2002, Emlen et al. 2005, Garamszegi et al. 2005, Ord & Martins 2006, Price et al. 2007, Puniamoorthy et al. 2008, Symonds et al. 2009), but the present study is, to our knowledge, the first comparative histological study on insect exocrine glands that are under sexual selection. Males of all but one of the investigated Philanthinae bear enormous and elaborate head glands that are considerably larger and more complex than in females of any species of this subfamily (Weiss et al. 2015). The exaggeration of the male glands emphasizes their significance for mate attraction and the strength of sexual selection acting upon them.

Our comprehensive investigation revealed considerable interspecific variation with numerous species deviating from their close relatives with regard to gland occurrence, size and morphology, as well as the incidence, specific type and arrangement of associated secretory cells. Nevertheless there was a clear phylogenetic trend in gland morphology (summarized in Figure 5.5). Ensuing from a plesiomorphic state, two main evolutionary trends emerge: First, the PPG increases in size and complexity and becomes involved in the production and storage of the marking secretion. Second, the MG, in return, decreases in size and is eventually completely lost.

### 5.5.1 The plesiomorphic state of the Philanthinae

About half of the species under study lacked an MG. To shed light on the plesiomorphic state of the subfamily, we conducted an ancestral state reconstruction. The most likely scenario is the presence of an MG in the predecessor of the Philanthinae, of the Philanthini and of the genus

*Philanthus* (including *Trachypus*) with repeated losses in single lineages and one gain (Figure 5.5, Figure S5.4). This view is corroborated by the fact that such ectal MGs (Fortunato et al. 2000, Pietrobon & Caetano 2003, Penagos-Arévalo et al. 2015) as found in male Philanthinae occur in all major lineages of the Aculeata (bees: e.g. Cruz-Landim 1967, Cruz-Landim et al. 2005, Galvani & Settembrini 2013; apoid wasps: Duffield et al. 1981, Penagos-Arévalo et al. 2015; vespid wasps: Pietrobon & Caetano 2003; ants: e.g. Blum 1969, do Amaral & Machado-Santelli 2008) and in parasitoid wasps (Stökl & Herzner 2016, Zimmermann & Vilhelmsen 2016). Moreover, females of all investigated Philanthinae have MGs, albeit small (Weiss et al. 2015), indicating that the genetic information to develop this gland is present throughout the subfamily. Males of all studied species possess a PPG that is probably homologous to the PPGs of ants (Formicidae) (Herzner et al. 2007a, Strohm et al. 2010) and the cockroach wasp *Ampulex compressa* (Ampulicidae) (Herzner et al. 2011b). In the majority of species under study, the PPG is associated with secretory cells. An ASR for the occurrence of these cells revealed that they were probably absent in the last common ancestor of Philanthinae and Philanthini. Accordingly, these cells must have evolved in the last common ancestor of *Philanthus*/*Trachypus*, but were lost several times within this taxon and regained at least once (Figure 5.5, Figure S5.5).

The inferred plesiomorphic state of male Philanthinae is represented by the investigated Cercerini and Aphilanthopsini with their large MGs and small PPG reservoirs devoid of secretory cells (Figure 5.5). The MGs of these species share a type of gland cells that bear end apparatus but, in contrast to typical NQ-class 3 cells, do not show canals. Such gland cells seem uncommon, but have been described for an ant (Billen et al. 2015) and some bee species (Galvani & Settembrini 2013). Notably, there is some variation among the Cercerini in that *C. rybyensis* males have an additional part of the MG with typical NQ-class 3 cells. Our results on *C. rybyensis* are largely consistent with Ågren (1977), who, however, did not mention the gland cells with end apparatus but no canals. The enormous size of the MG reservoir of male Cercerini and Aphilanthopsini and the high number of associated secretory cells suggest that the function of the MG comprises both production and storage of the male marking secretion. In other taxa of Hymenoptera, the MG is known as source of different pheromones like male and female sex pheromones (Vinson et al. 1982, 1984, Hefetz 1983, Ayasse et al. 2001, Stökl & Herzner 2016), the queen pheromone in honeybees (*A. mellifera*) (e.g. Slessor et al. 1988, Winston & Slessor 1992) and alarm pheromones in different ants (e.g. Hughes et al. 2001a, 2001b, Lalor & Hughes 2011). The MG can also be the source of defensive secretions in parasitoid wasps (Stökl & Herzner 2016), bees (Cane & Michener 1983, Cane et al. 1983), and ants (Chadha et al. 1962).

The PPGs found in male Cercerini and Aphilanthopsini in the present study largely resemble the PPGs of the respective conspecific females (Weiss et al. 2015). Moreover, the shape and structure is quite similar to the PPGs of both sexes of the cockroach wasp *A. compressa* (Herzner et al. 2011b), a rather basal taxon within the Apoidea (Melo 1999, Debevec et al. 2012). Notably, a PPG had not previously been described for male Cercerini and Aphilanthopsini and currently no information is available on their chemistry. Considering their small size and the lack of secretory cells, we hypothesize that in these tribes the males' PPGs do not play an important role in the production and/or storage of a marking secretion. Instead, as suggested for *A. compressa* (Herzner et al. 2011b), the PPG may function as a hydrocarbon reservoir. Until recently, a PPG was only known from ants where it mainly serves to generate the colony odor that is also based on hydrocarbons (Soroker et al. 1994, 1995, Hefetz et al. 1996, Lenoir et al. 1999; for a review of other functions see Eelen et al. 2006). Such a "social function" of the PPG can be ruled out for the solitary Cercerini and Aphilanthopsini.

### 5.5.2 The involvement of the PPG

The head glands of male Philanthini differ markedly from the Cercerini and Aphilanthopsini since their MGs are more or less reduced and their PPGs are typically considerably larger and more complex (Figure 5.5). Like in most Hymenoptera (e.g. Cruz-Landim & Reginato 2001, Grasso et al. 2004, Billen et al. 2013, Boonen et al. 2013, Stökl & Herzner 2016, Zimmermann & Vilhelmsen 2016), the MGs of male Philanthini are exclusively associated with typical NQ-class 3 cells, either in single units or arranged in acini. As in *P. triangulum* (Kroiss et al. 2006, Herzner et al. 2007a, Goettler & Strohm 2008), the male MG of other Philanthini is presumably also involved in the production of the marking pheromone.

Taking into account its position at the very base of the Philanthini, the genus *Philanthinus* may be expected to represent an intermediate state between the Cercerini and Aphilanthopsini and the Philanthini. In fact, the somewhat smaller MG with typical NQ-class 3 gland cells and the large PPG of *P. quattuordecimpunctatus* (Figure 5.3) support this view. However, in contrast to most *Philanthus* species its PPG is not associated with secretory cells (Figure 5.5) and *P. quattuordecimpunctatus* stands out from all other Philanthinae with regard to the structural organization of the PPG in lamella-like branches.

In the genus *Philanthus*, males of nearly all studied species possess at least moderately large PPGs (Figure 5.5, see also Figure 5.3). In most species these PPGs shows extensive ramifications that are

closely associated with cells (syncytia or, rarely, cell aggregations) that show clear signs of secretory activity, like large nuclei with several nucleoli and numerous vesicles. Even though these cells do not conform to any previously described type of secretory cell (Noirot & Quennedey 1974, Billen 2009, 2011), we hypothesize that they synthesize compounds of the marking secretion that are transferred to the PPG reservoir, where they are stored until release during territory marking. How these cells evolved and whether their secretion is transported to the PPG lumen by direct contact as suggested by their close proximity to the PPG ramifications is not known yet. Notably, in species that have lost these secretory cells associated with the PPG (*P. t. triangulum*, *P. t. diadema, P.* cf. *basalis*, *P. capensis*, *T. elongatus*, and *T. patagonensis*) the PPG reservoirs consist of voluminous tubes without ramifications that presumably merely store the marking secretion that is produced in the MG (Kroiss et al. 2006, Herzner et al. 2007a, Goettler & Strohm 2008). Inspection of the mapping of PPG characters on the phylogeny suggests that the secretory cells and the elaboration of the PPG reservoir may have evolved concurrently at the base of the genus *Philanthus* (Figure 5.5).

Our results suggest that the PPG contributes to a variable degree to pheromone storage and production in males of most Philanthini. So the question arises why and how its involvement in scent marking came about. Beewolf females have been observed to simply alight in a male's territory and allow mating without additional courtship by males (Evans & O'Neill 1988, Kroiss et al. 2010). Therefore, the conspicuousness of the territory, mediated by the composition and amount of marking pheromone, is probably the most important determinant for male reproductive success. Moreover, the spatial proximity of scent marking males in leks, as has been shown for several *Philanthus* species (Evans & O'Neill 1988, O'Neill 2001, Kroiss et al. 2010), might allow females to directly compare territories and their owners. This results in strong sexual selection on males to maximize both the quantity and quality of the marking secretion.

The original dual role of the MG as site of synthesis and reservoir of the marking secretion (as found in the Cercerini and Aphilanthopsini) might have limited the ability of males to synthesize and store larger amounts of marking secretion or to add novel compounds to the blend. For example, novel classes of compounds might have interfered with the synthesis or storage of the existing components (e.g. due to chemical reactions between acids and alcohols), thus promoting the evolution of novel secretory cells and a separate reservoir. The first evolutionary step towards its prominent role in scent marking might thus have been a minor participation of the PPG in the storage and production of the marking secretion. Ongoing selection on pheromone quantity and quality would subsequently have enlarged the PPG and augmented its contribution. Whether the

involvement of the PPG to pheromone production is accompanied by changes in the chemical composition of marking secretions in the Philanthini, in particular by the addition of novel classes of compounds, should be revealed by a comparative study of the marking secretions among the Philanthini.

An increase in the amount of scent marking secretion would clearly have been an advantage for mate attraction (Droney & Hock 1998, Ruther et al. 2009, Foster & Johnson 2011). The addition of novel components to a sex pheromone, however, may represent a saltational evolutionary change (Symonds & Elgar 2008), potentially even hindering mate recognition. Novel compounds might nevertheless be selected for by several not mutually exclusive causes like predation avoidance, male-male competition, and female choice (Haynes & Yeargan 1999, Raffa et al. 2007, Symonds & Elgar 2008). There is currently no evidence that male scent marking in the Philanthini is effective in repelling predators or in keeping conspecific males at bay. However, different processes related to female choice might explain the evolution of novel pheromone components. First, female sensory biases (Morris 1998, Wiens 2001, Palmer et al. 2005, Elias et al. 2006) that evolved for prey recognition purposes might influence pheromone composition as in *P. triangulum* (Herzner et al. 2005, Schmitt et al. 2007, Steiger et al. 2010). Consequently, a shift in the females' prey spectrum might select for changes in the males' marking secretion. Second, Fisher-Zahavi processes (Prum 1997, Kokko et al. 2002, 2003) could cause the addition of novel components. In Fisher's run-away model a female preference might arise accidentally and coevolve with the preferred trait; but this process has rarely been considered for pheromone evolution. Female choice for good or compatible genes could affect the evolution of pheromones (Johansson & Jones 2007), in that new components could indicate additional aspects of male quality (Herzner et al. 2006) or improve signal reliability (Mahr et al. 2016). Finally, since sympatry is widespread among *Philanthus* species (e.g. Evans & O'Neill 1988; G. Herzner, E. Strohm, M. Kaltenpoth, *unpublished data*) the establishment or reinforcement of reproductive isolation between species (Symonds & Elgar 2008, Smadja & Butlin 2009, Niehuis et al. 2013, Weber et al. 2016) might have selected for novel pheromone components (Johansson & Jones 2007).

If the involvement of the PPG enhanced mate attraction in male Philanthini, the question arises why the PPG did not get involved in scent marking (and was not enlarged) in the Cercerini and Aphilanthopsini as well. One possible explanation is that males of these tribes experience weaker sexual selection because, compared to male Philanthini, they have less pronounced territorial behavior and are spatially more dispersed (Alcock 1975, Steiner 1978, Evans & O'Neill 1985, 1988, Evans 2000). Different intensities of sexual selection on males could also explain that PPG

morphology shows a conspicuously congruent pattern in both sexes among the Philanthinae, with smaller PPGs in the Cercerini and Aphilanthopsini and larger, more complex PPGs in the Philanthini (Weiss at el. 2015). Owing to correlated evolution between the sexes (Lande 1980, Amundsen 2000, Potti & Canal 2011, Tobias et al. 2012), genetic changes underlying the sexually selected elaboration of the PPG in male Philanthini, as documented in this study, could have facilitated an enlargement of the PPG and the evolution of prey embalming in female Philanthini (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2007b, 2011a, 2013, Weiss et al. 2015). That the PPG has evolved independently in males and females and the observed congruency across tribes is merely accidental seems rather unlikely. Yet, another plausible scenario is that the initial augmentation of the PPG might first have evolved in female Philanthini due to strong natural selection for prey embalming (Strohm & Linsenmair 2001, Herzner & Strohm 2007, Herzner et al. 2007b, 2011a, 2013, Weiss et al. 2015) and, again assuming correlated evolution between the sexes (Lande 1980, Amundsen 2000, Potti & Canal 2011, Tobias et al. 2012), the resulting genetic changes could have facilitated the subsequent enlargement and elaboration of the PPG in male Philanthini by sexual selection. Different natural selection pressures on female Cercerini and Aphilanthopsini (Weiss et al. 2015) may have kept the PPGs of both sexes of these basal tribes comparably small and simple.

### 5.5.3 The loss of the MG

The loss of the MG in the Nearctic *Philanthus* was surprising, since MGs had previously been reported from males of five of these species (Gwynne 1978, Schmidt et al. 1985, McDaniel et al. 1987, 1992). We suspect that in these studies the large PPGs were mistaken for MGs, because their conclusions were based on dissections that hardly allow the discrimination of the two glands and at that time PPGs were only known from ants (Herzner et al. 2007a, Kroiss et al. 2006).

Notably, in all but one species without MG the PPGs are huge and show extensive ramifications in direct contact with multinuclear syncytia (or aggregations of cells in *P. venustus*) (Figure 5.5). Only in species lacking the MG (and in *P. multimaculatus*) the PPG is interspersed with conspicuous small cells (Figure 5.5, see also Figure 5.2 D and F). This might suggest that these cells substitute for some function of the MG. However their small size and little cytoplasm contradict a secretory capacity. Due to the size and complexity of the PPGs and their association with large secretory cell clusters, we conclude that in the species without MGs, the PPG alone is responsible for the synthesis and storage of the marking secretion.

While it appears plausible that the enlargement of the PPG caused a reduction of the MG, its complete loss in several clades of the Philanthini is most puzzling, because it might have been accompanied by the loss of certain components of the marking secretion. Non-adaptive explanations like genetic drift in small populations could hardly explain the disappearance of a whole gland system. According to the above mentioned idea that the involvement of different glands is driven by hybridization avoidance, the loss of components of a sex pheromone and the respective gland might be possible if the risk of hybridization is lowered. However, since particularly Nearctic species often occur in sympatry (e.g. Evans & O'Neill 1988; G. Herzner, E. Strohm, M. Kaltenpoth, *unpublished data*), a reduced risk of hybridization compared to other clades seems unlikely. An alternative explanation is that a change in female preferences to compounds that can be more efficiently produced in the PPG might make an MG superfluous. Female preferences (Morris 1998, Wiens 2001, Palmer et al. 2005, Elias et al. 2006) might be altered because of a change in their prey spectrum as explained above. In many Nearctic *Philanthus*, females prey not only on bees but also on wasps, whereas the latter habit seems to be rare in Palearctic and Afrotropical species (Evans & O'Neill 1988). Whether such a difference could cause the loss of the MG in males of the Nearctic species cannot be answered yet. Otherwise, there are no conspicuous differences between the Nearctic species and their Palearctic/Afrotropical congeners with regard to scent marking and reproductive behavior (Evans & O'Neill 1988) that could explain the loss of the MG. Unfortunately, very little is known about the other two species without MG, *P. venustus* and *P. pulcherrimus*.

The loss of a sexual character is becoming increasingly recognized as a common event in the evolution of sexually selected traits and may have different causes (Wiens 2001, Porter & Crandall 2003). In beewolves, however, the actual trait, scent marking, persists while the source of the secretion is changed. A similar phenomenon has been reported for solitary bees of the genus *Centris*. Depending on the species, males scent mark territories with a secretion from either the MG or tibial glands and the respective other gland is reduced (Vinson et al. 1982, 1984, Williams et al. 1984).

### 5.5.4 Taxa deviating from the overall trend

*Philanthus albopilosus* is the only known species of the genus in which males do not establish and scent mark territories (Evans & O`Neill 1988). Therefore, they do not need the respective glands anymore and their PPG has been reduced (Figure 5.5). This provides indirect evidence for the role of the PPG in the production and storage of the marking secretion in other male Philanthini. The

reduction of a gland following the loss of its function has been reported for fungus-growing ants. In monandrous attine ants, males transfer an antiaphrodisiac from accessory glands during copulation; in polyandrous species, however, males do not mark mated queens and their accessory glands were reduced or completely lost (Mikheyev 2004).

The regain of the MG in males of the Nearctic *P. multimaculatus* (Figure 5.5) is puzzling since there are no conspicuous differences to its Nearctic congeners with regard to their territorial behavior (Evans & O'Neill 1988). Also, why in some species (*P. triangulum, P.* cf. *basalis, P. capensis*) the secretory cells of the PPG were lost while the reservoir became the main storage organ (Figure 5.5) cannot be answered yet.

### 5.5.5 Conclusion

There is substantial evidence that sexually selected traits can undergo rapid evolutionary change, including losses and gains (Meyer et al. 1994, Meyer 1997, Kimball et al. 2001, Kopp & True 2002). In particular the Fisher-Zahavi processes (Prum 1997, Kokko 2002, 2003) as well as sexual antagonism, like chase-away selection (Holland & Rice 1998) and female sensory biases (Morris 1998, Wiens 2001, Palmer et al. 2005, Elias et al. 2006) might cause complex phylogenetic patterns in sexually selected characters (e.g. Pomiankowski & Iwasa 1998, Omland & Lanyon 2000, Price & Lanyon 2004, Kopp & True 2002, Emlen et al. 2005, Price et al. 2007, Ord & Martins 2006). Our comparative morphological analyses of male head glands revealed extensive interspecific variation within the Philanthinae, in particular among the Philanthini. While we found clear phylogenetic trends, there are also intriguing deviations and reversals (Figure 5.5). The glands of female Philanthini, by contrast, appear virtually uniform with mostly only gradual variation and no loss of a gland system or the addition of novel components like secretory cells (Weiss et al. 2015), probably as a result of stabilizing natural selection. Other evolutionary forces like genetic drift and mutations should affect males and females similarly and can therefore be excluded as causes for the observed higher diversity among males. Taken together our findings support the hypothesis that strong sexual selection acting on male pheromone glands has led to rapid evolutionary changes and to a substantially higher interspecific morphological diversity in males than in females. Taking into account that about 135 of the ca. 170 described species of Philanthini (Pulawski 2016a) have not been investigated so far, the high diversity observed in this study suggests that there are probably more species with unique and novel gland characteristics yet to be discovered. Further studies on the chemical composition of the marking secretions, male territorial behavior, mate attraction as well as female prey spectrum and mate choice will help to

unravel the ecological and evolutionary causes that have given rise to the remarkable diversity and phylogenetic trends in male head gland morphology among the Philanthinae.

## 5.6 Supplementary material

The following sections provide the information given in Additional File 1 (sections 5.6.1 through 5.6.4) and Additional File 2 (sections 5.6.5 through 5.6.7) of the accepted manuscript.

### 5.6.1 Definition of morphological characters and coding of character states and phylogenetic/ phylogeographic affiliation

Based on the comprehensive examination of both semithin histological sections and 3D-reconstructions of the head glands of males of 33 species and one subspecies of the Philanthinae (Table 5.1), we defined 14 morphological characters for the PPG and MG, as well as associated secretory cells. For each character, different states were categorized and numerically coded for the statistical analysis as described below. Note that for characters 2, 4, 5, 10, and 12 the character states can be ranked and thus represent ordinal data. Moreover, for all statistical analyses for characters 3, 4, 9, and 13 some categories were pooled as specified below ('combined dataset', Table S5.2; see also section 5.3.4.2).

**PPG:**

1 *Overall structure of the PPG reservoir*.

**(0)** PPG consists of both an upper reservoir originating from the dorsal side of the pharynx and a lower evagination originating ventrally from the pharynx

**(1)** PPG consists of only the upper reservoir

2 *Relative size of the upper PPG reservoir*. In male Philanthinae, the paired reservoirs of the upper PPG originate dorsally from the pharynx and extend backwards subjacent to the cuticle of the head capsule. In species with large PPGs, the reservoirs reach around the brain and may even proceed to the front of the head capsule ventrally to the brain, in some cases almost reaching the mandible base. The size of the upper PPG reservoir is specified as its longitudinal extension in relation to the size of the head capsule. This estimate is only slightly altered by the filling status of the gland, since this mainly affects the lateral extension of the gland. After a preliminary analysis we defined three size classes. The limits of these size classes follow a geometric progression with

the ranges of the size classes increasing by the factor 2. This allowed for a simple and reliable classification and covered the spectrum of cases.

**(0)** small PPG (spanning approximately 25 - 50% of the head capsule)

**(1)** medium sized PPG (spanning approximately 50 - 100% of the head capsule)

**(2)** large PPG (spanning approximately 100 - 200% of the head capsule, i.e. it reaches around the brain and extends downwards towards the mandible base ventrally to the brain)

3 *Modifications of the PPG morphology*. In all investigated male Philanthinae the main part of the PPG comprises paired lateral reservoirs of the upper gland part extending dorsally above and, depending on its size, around the brain (dPPG in Figure 5.1). In most species these reservoirs are accompanied by a second (often smaller) set of paired reservoirs branching off from the dorsal reservoirs and extending somewhat lateral and anterior to the brain (aPPG in Figure 5.1). Some species possess an additional considerably smaller unpaired part of the PPG consisting of a ventral evagination of the pharynx (see character 1). However, most species show modifications of this basic pattern that could be clearly differentiated. We distinguished six such morphological modifications of the PPG, which were denoted A - F and are described in Figure S5.1. The species under study exhibited different combinations of these modifications. For the 'combined dataset', categories (7) and (8) were pooled (Table S5.2).

**(0)** A

**(1)** A + E

**(2)** A + F

**(3)** A + B

**(4)** A + B + E + F

**(5)** A + B + C

**(6)** A + B + C + F

**(7)** A + B + C + E + F

**(8)** B + D + E + F

4 *Branching of the PPG reservoir*. The main reservoir of the upper PPG can be branched to a variable extent, ranging from unbranched reservoirs, not associated with gland cells, to extensively branched reservoirs with numerous fine branches, which are directly associated with aggregations of mononuclear cells or syncytia of secretory cells (see character 7). For the 'combined dataset', categories (2) - (4) were pooled (Table S5.2).

**(0)** unbranched

**(1)** few voluminous branches (without fine branches)

**(2)** some fine branches

**(3)** many fine branches

**(4)** very many fine branches

5 *Number of openings of the lower part of the PPG to the pharynx*.

**(0)** one opening

**(1)** two openings

6 *Structure of the inner walls of the PPG reservoir*.

**(0)** unstructured

**(1)** cuticular ripples

**(2)** hairs

7 *Type of gland cells associated with the PPG*. In 23 of the investigated 33 species of Philanthinae the PPG reservoir is associated with cells with a presumably secretory function. However, these cells do not resemble any of the gland cells classes as defined by Noirot and Quennedey (1974). These cells could be assigned to clearly definable categories.

**(0)** no gland cells

**(1)** PPG-type 1: several gland cells are tightly associated and form cell aggregations that are directly associated with very fine branches of the PPG (see character 4)

**(2)** PPG-type 2: similar to PPG-type 1, but the cell aggregations are interspersed with small rounded cells (Figure 5.2 D)

**(3)** PPG-type 3: the secretory cells consist of syncytia (i.e. aggregations of cells but no cell membranes visible between cells) that are directly associated with very fine branches of the PPG (see character 4) (Figure 5.2 E)

**(4)** PPG-type 4: similar to PPG-type 3 but the syncytia are interspersed with small rounded cells (similar to those described for PPG-type 2) (Figure 5.2 F)

In the preliminary investigation, we also recorded the number of openings of the upper part of the PPG to the pharynx. However, this character was invariant with all investigated species possessing a separate opening for each of the two sides of the upper PPG and was therefore not included in the dataset.

**MG:**

8 *Presence of the MG reservoir*.

**(0)** no MG reservoir

**(1)** MG reservoir present

9 S*tructure of the MG reservoir*. In species of the tribe Philanthini, the MG can consist of an upper and a lower reservoir, which open dorsally and ventrally to the mandible, respectively. In male

Cercerini and Aphilanthopsini, the MG likewise originates dorsally to the mandible, but possesses a thinner and less structured wall as compared to the MG of the Philanthini. In one species, *Cerceris rybyensis*, this thin-walled MG reservoir occurs alongside a small reservoir resembling the upper MG of the Philanthini. We regarded the thin-walled upper MG of the Cercerini and Aphilanthopsini as a distinct part of the MG (categories (0) and (1) in the 'full dataset', Table S5.1). For the 'combined dataset', however, categories (0) and (1) were pooled with category (2) (Table S5.2).

**(0)** only the thin-walled MG reservoir is present

**(1)** in addition to the thin-walled MG (0), a second MG reservoir with thicker walls (like in (2)-(4)) is present

**(2)** only the upper part of the MG reservoir is present

**(3)** only the lower part of the MG reservoir is present

**(4)** both upper and lower parts of the MG is reservoir present

10 *Relative size of the MG reservoir*. The MG extends from the mandible base laterally and dorsally throughout the head capsule, either proximal or lateral to the PPG reservoir. In some cases, the MG reaches around the brain and can even extend to the front part of the head capsule ventrally to the brain. Hence, analogous to the PPG, we determined the size of the MG relative to the head capsule.

**(0)** small MG (spanning approximately 25 - 50% of the head capsule)

**(1)** medium sized MG (spanning approximately 50 - 100% of the head capsule)

**(2)** large MG (spanning approximately 100 - 200% of the head capsule, i.e. it reaches around the brain and extends downwards towards the mandible base ventrally to the brain)

11 *Location of the MG reservoir in the head capsule*. Depending on its size (see character 10) and on which part of the gland is developed (see character 9), parts of the MG can either be located only in the front part of the head capsule anterior to the brain (A), reach further into the head capsule dorsally to the brain (D), cover the full extension of the head capsule ending behind the brain (B), and/or extend ventral around the brain (V).

**(0)** A

**(1)** A + D

**(2)** A + D + B

**(3)** A + D + B + V

12 *Branching of the MG reservoir*.

**(0)** unbranched: the MG consists of paired sac-like evaginations as reservoirs

**(1)** low: the MG consists of paired reservoirs, each with a few (2-5) distinct branches

**(2)** high: the MG consists of paired reservoirs, each with many (>5) branches

13 *Structure of the inner walls of the MG reservoir*. For the 'combined dataset', categories (2) - (4) were pooled (Table S5.2).

**(0)** unstructured

**(1)** bearing scattered thin hairs

**(2)** bearing many thick hairs

**(3)** bearing several hairs that jointly originate from the tops of flat cuticular sockets, giving the impression of small bushes in the sagittal semithin sections

**(4)** bearing several ramified hairs that originate laterally from pointed cuticular spines, giving the impression of small trees in the sagittal semithin sections

14 *Type of gland cells associated with the MG*. In all of the 19 investigated species possessing an MG, the reservoir is associated with gland cells. These cells can be clearly assigned to one of several categories. As stated in section 5.3.2, we will refer to gland cells corresponding to one of the classes defined by Noirot and Quennedey (1974) as 'NQ-class' cells, but we found additional gland cell types that did not match these definitions.

**(0)** MG-type 1: single gland cells, showing end apparatus but no canals (Figure 5.2 C)

**(1)** MG-type 2: single typical NQ-class 3 gland cells with end apparatus and canal (Figure 5.2 A)

**(2)** MG-type 3: same as MG-type 2, but arranged in acini, i.e. several NQ-class 3 cells are tightly arranged in cell clusters and jointly connected to the MG reservoir via a bundle of canals (Figure 5.2 B)

**(3)** MG-type 4: MG-type 1 and MG-type 2 cells associated with different parts of the MG

**Supplementary variable for categorical principle components analysis (CATPCA):**

*Phylogenetic/phylogeographic affiliation.* The subfamily Philanthinae consist of eight genera separated into the three tribes Cercerini, Aphilanthopsini, and Philanthini (Alexander 1992). According to a recent molecular phylogeny (Kaltenpoth et al. 2014), the tribe Philanthini can be divided into several monophyletic clades, largely reflecting their geographic distribution: The genus *Philanthinus* forms a basal sister clade to the Palearctic, Afrotropical, and Indian species of the genus *Philanthus*, from which the Nearctic *Philanthus* species, as well as the Neotropical genus *Trachypus* branch off (thus, *Trachypus* constituting a monophyletic sister group of the Nearctic *Philanthus*, rather than a separate genus, Kaltenpoth et al. 2014). The general phylogenetic affiliation of each species and the geographic origin of species of the Philanthini were coded accordingly (see also dendrograms in Figures S5.4 and S5.5, section 5.6.4.3) and included into the CATPCA as a supplementary variable (see section 5.6.2.1 below).

**(0)** tribe Cercerini: genus *Cerceris*

**(1)** tribe Aphilanthopsini: genus *Clypeadon*

**(2)** tribe Philanthini: genus *Philanthinus*

**(3)** tribe Philanthini: basal Palearctic/Indian *Philanthus* clade

**(4)** tribe Philanthini: main Palearctic/Afrotropical *Philanthus* clade

**(5)** tribe Philanthini: Nearctic *Philanthus* species

**(6)** tribe Philanthini: genus *Trachypus*

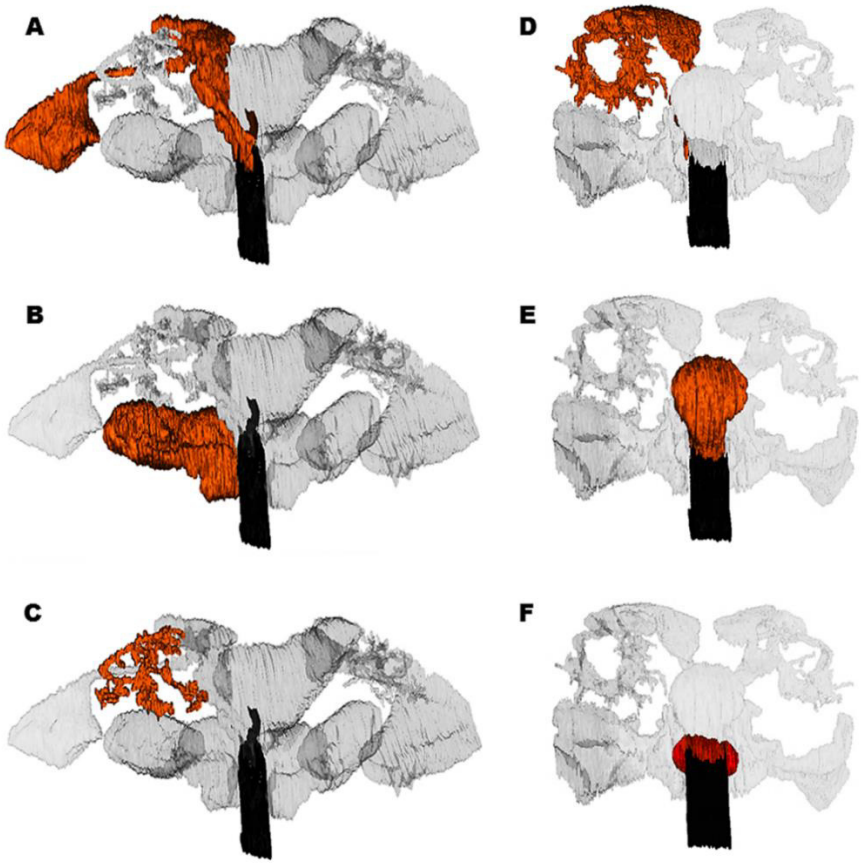


**Figure S5.1** Morphological modifications of the postpharyngeal gland (PPG) of male Philanthinae. The PPG of males of all investigated Philanthinae is made up of different combinations of six distinct morphological elements (see character 3 in section 5.6.1 above), which are illustrated here by examples of the PPGs of the Afrotropical *Philanthus rugosus* (A - C) and the Nearctic *Philanthus politus* (D - F) [whole PPG shown in light grey, in each panel the described element is highlighted in either orange (upper PPG) or red (lower PPG, panel F). Note that, with the exception of (E) and (F), the described elements are paired structures. **(A)** Unbranched tube-shaped reservoir, directly originating from the pharynx (black) and extending dorsally subjacent to the cuticle of the head capsule (dPPG in Figure 5.1); the size of this part can vary considerably among species. **(B)** Unbranched, tube- or sac-shaped reservoir originating from (A) and extending laterally anterior to the brain (aPPG in Figure 5.1); also this part of the PPG shows considerable interspecific size variation. **(C)** Fine branches originating from (A) and subsequently ramifying before reaching one or several of the associated secretory cells (not shown) (see also character 7 in section 5.6.1 above). **(D)** Gland reservoir originating dorsally from the pharynx and extending posterior around the brain associated with short branches, thus resembling a combination of (A) and (C) (dPPG in Figure 5.1); yet, unlike (A) the reservoir has a very specific coiled appearance and, unlike (C), the fine, comparatively short branches originate in regular intervals and do not further ramify before reaching associated secretory cells (not shown). **(E)** Unpaired, sac-like dorsal evagination at the opening of the PPG to the pharynx. **(F)** Small, unbranched ventral evagination of the pharynx (referred to as 'lower PPG' in the text).

**Table S5.1** 'Full dataset' of the morphological characters as recorded in the comparative analysis of head gland morphology of male Philanthinae. A total of 14 morphological characters of the postpharyngeal gland (PPG) and the mandibular gland (MG) of 33 species and one subspecies were defined. Species IDs correspond to Table 5.1. The numbering of the characters and the numeric coding of the character states correspond to the description in section 5.6.1. (?) character state could not be determined, (-) character not present in this species.

| | | PPG | | | | | | | MG | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Species** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| **1** | ***Cerceris quinquefasciata*** | 1 | 0 | 0 | 0 | - | 1 | 0 | 1 | 0 | 2 | 3 | 2 | 1 | 0 |
| **2** | ***Cerceris rybyensis*** | 1 | 1 | 0 | 0 | - | 1 | 0 | 1 | 1 | 2 | 2 | 1 | 1 | 3 |
| **3** | ***Clypeadon laticinctus*** | 0 | 1 | 2 | 0 | 1 | 1 | 0 | 1 | 0 | 2 | 2 | 1 | 0 | 0 |
| **4** | ***Philanthinus quattuordecimpunctatus*** | 0 | 1 | 4 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 1 | 0 |
| **5** | ***Philanthus* cf. *basalis*** | 1 | 2 | 3 | 1 | - | ? | 0 | 1 | 4 | 2 | 1 | 0 | 1 | 2 |
| **6** | ***Philanthus pulcherrimus*** | 0 | 2 | 5 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **7** | ***Philanthus spec* (India)** | 1 | 2 | 5 | 4 | - | 0 | 3 | 1 | 2 | 1 | 1 | 0 | 3 | 2 |
| **8** | ***Philanthus venustus*** | 0 | 2 | 7 | 4 | 0 | 2 | 2 | 0 | - | - | - | - | - | - |
| **9** | ***Philanthus capensis*** | 1 | 2 | 3 | 1 | - | 2 | 0 | 1 | 2 | 2 | 1 | 1 | 2 | 2 |
| **10** | ***Philanthus coronatus*** | 1 | 2 | 5 | 3 | - | 0 | 3 | 1 | 2 | 1 | 1 | 0 | 2 | 2 |
| **11** | ***Philanthus fuscipennis*** | 1 | 2 | 5 | 2 | - | ? | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| **12** | ***Philanthus histrio*** | 1 | 2 | 5 | 4 | - | 2 | 3 | 1 | 2 | 0 | 0 | 0 | 3 | 1 |
| **13** | ***Philanthus loefflingi*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| **14** | ***Philanthus melanderi*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 2 | 1 | 1 | 0 | 2 | 2 |
| **15** | ***Philanthus rugosus*** | 1 | 2 | 5 | 4 | - | 2 | 3 | 1 | 2 | 0 | 0 | 0 | 3 | 1 |
| **16** | ***Philanthus triangulum triangulum*** | 1 | 2 | 3 | 1 | - | 1 | 0 | 1 | 4 | 2 | 1 | 0 | 4 | 2 |
| **17** | ***Philanthus triangulum diadema*** | 1 | 2 | 3 | 1 | - | 1 | 0 | 1 | 4 | 2 | 1 | 0 | 4 | 2 |
| **18** | ***Philanthus albopilosus*** | 1 | 1 | 0 | 1 | - | 0 | 0 | 0 | - | - | - | - | - | - |
| **19** | ***Philanthus barbiger*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **20** | ***Philanthus bicinctus*** | 0 | 2 | 7 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **21** | ***Philanthus crotoniphilus*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **22** | ***Philanthus gibbosus*** | ? | ? | ? | ? | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **23** | ***Philanthus gloriosus*** | 0 | 2 | 7 | 2 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **24** | ***Philanthus multimaculatus*** | 0 | 2 | 8 | 3 | 0 | 0 | 4 | 1 | 3 | 0 | 0 | 0 | 0 | 1 |
| **25** | ***Philanthus occidentalis*** | ? | 2 | ? | 4 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **26** | ***Philanthus pacificus*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **27** | ***Philanthus parkeri*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **28** | ***Philanthus politus*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **29** | ***Philanthus psyche*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **30** | ***Philanthus pulcher*** | 0 | 2 | 8 | 4 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **31** | ***Philanthus ventilabris*** | 0 | 2 | ? | 1 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **32** | ***Trachypus elongatus*** | 1 | 2 | 3 | 1 | - | 0 | 0 | 1 | 3 | 1 | 0 | 0 | 1 | 1 |
| **33** | ***Trachypus flavidus*** | 1 | 2 | 5 | 2 | - | 0 | 1 | 1 | ? | 0 | 0 | ? | 0 | 1 |
| **34** | ***Trachypus patagonensis*** | ? | ? | ? | ? | ? | ? | 0 | 1 | 4 | 1 | 0 | ? | 0 | 1 |

**Table S5.2** 'Combined dataset' used for the statistical analyses of head gland morphology of male Philanthinae. For the four characters shaded in grey, some character states were pooled (see description in section 5.6.1 above) as compared to the 'full dataset' (Table S5.1). Note that *T. patagonensis* (ID 34) has been excluded from this dataset since for most of the characters no reliable information could be obtained (Table S5.1 and section 5.3.4.2). Species IDs correspond to Table 5.1. The numbering of the characters and the numeric coding of the character states correspond to the description in section 5.6.1. (?) character state could not be determined, (-) character not present in this species.

| | | PPG | | | | | | | MG | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Species** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| **1** | ***Cerceris quinquefasciata*** | 1 | 0 | 0 | 0 | - | 1 | 0 | 1 | 0 | 2 | 3 | 2 | 1 | 0 |
| **2** | ***Cerceris rybyensis*** | 1 | 1 | 0 | 0 | - | 1 | 0 | 1 | 0 | 2 | 2 | 1 | 1 | 3 |
| **3** | ***Clypeadon laticinctus*** | 0 | 1 | 2 | 0 | 1 | 1 | 0 | 1 | 0 | 2 | 2 | 1 | 0 | 0 |
| **4** | ***Philanthinus quattuordecimpunctatus*** | 0 | 1 | 4 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 2 | 0 | 1 | 0 |
| **5** | ***Philanthus* cf. *basalis*** | 1 | 2 | 3 | 1 | - | ? | 0 | 1 | 2 | 2 | 1 | 0 | 1 | 2 |
| **6** | ***Philanthus pulcherrimus*** | 0 | 2 | 5 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **7** | ***Philanthus spec* (India)** | 1 | 2 | 5 | 2 | - | 0 | 3 | 1 | 0 | 1 | 1 | 0 | 2 | 2 |
| **8** | ***Philanthus venustus*** | 0 | 2 | 7 | 2 | 0 | 2 | 2 | 0 | - | - | - | - | - | - |
| **9** | ***Philanthus capensis*** | 1 | 2 | 3 | 1 | - | 2 | 0 | 1 | 0 | 2 | 1 | 1 | 1 | 2 |
| **10** | ***Philanthus coronatus*** | 1 | 2 | 5 | 2 | - | 0 | 3 | 1 | 0 | 1 | 1 | 0 | 1 | 2 |
| **11** | ***Philanthus fuscipennis*** | 1 | 2 | 5 | 2 | - | ? | 3 | 1 | 0 | 1 | 1 | 1 | 1 | 2 |
| **12** | ***Philanthus histrio*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 2 | 1 |
| **13** | ***Philanthus loefflingi*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 0 | 1 | 1 | 1 | 1 | 2 |
| **14** | ***Philanthus melanderi*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 1 | 2 |
| **15** | ***Philanthus rugosus*** | 1 | 2 | 5 | 2 | - | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 2 | 1 |
| **16** | ***Philanthus triangulum triangulum*** | 1 | 2 | 3 | 1 | - | 1 | 0 | 1 | 2 | 2 | 1 | 0 | 2 | 2 |
| **17** | ***Philanthus triangulum diadema*** | 1 | 2 | 3 | 1 | - | 1 | 0 | 1 | 2 | 2 | 1 | 0 | 2 | 2 |
| **18** | ***Philanthus albopilosus*** | 1 | 1 | 0 | 1 | - | 0 | 0 | 0 | - | - | - | - | - | - |
| **19** | ***Philanthus barbiger*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **20** | ***Philanthus bicinctus*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **21** | ***Philanthus crotoniphilus*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **22** | ***Philanthus gibbosus*** | ? | ? | ? | ? | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **23** | ***Philanthus gloriosus*** | 0 | 2 | 7 | 2 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **24** | ***Philanthus multimaculatus*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| **25** | ***Philanthus occidentalis*** | ? | 2 | ? | 2 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **26** | ***Philanthus pacificus*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **27** | ***Philanthus parkeri*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **28** | ***Philanthus politus*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **29** | ***Philanthus psyche*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **30** | ***Philanthus pulcher*** | 0 | 2 | 7 | 2 | 0 | 0 | 4 | 0 | - | - | - | - | - | - |
| **31** | ***Philanthus ventilabris*** | 0 | 2 | ? | 1 | ? | 0 | 4 | 0 | - | - | - | - | - | - |
| **32** | ***Trachypus elongatus*** | 1 | 2 | 3 | 1 | - | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| **33** | ***Trachypus flavidus*** | 1 | 2 | 5 | 2 | - | 0 | 1 | 1 | ? | 0 | 0 | ? | 0 | 1 |

### 5.6.2 Statistical analysis of male gland morphology

#### *5.6.2.1 Categorical principal components analysis*

Analogously to a linear principal components analysis, CATPCA is a data mining tool to search for patterns of variation in a large set of (more or less correlated) variables by reducing them to a small number of uncorrelated principal components, thus facilitating their interpretation while retaining as much as possible of the information in the original data (Meulman et al. 2004b, Linting et al. 2007). Yet a CATPCA has two important advantages over its linear equivalent. As the initial analysis step of the CATPCA is the so called nonlinear optimal scaling transformation of the subjected variables, this method can be used to analyze ordinal and nominal variables by replacing the discrete categories of these variables with numeric values called category quantifications. Moreover, depending on the specified analysis level of the transformation, it can also detect nonlinear relationships between variables (Meulman et al. 2004b, Linting et al. 2007, Linting & van der Kooij 2012).

The 'full dataset' (Table S5.1) contains all character states as they were recorded. In the 'combined dataset' (Table S5.2) that was used for statistical analyses, we pooled character states wherever reasonable. Actually, the four characters 3 (Modifications of PPG morphology), 4 (Branching of the PPG), 9 (Overall structure of the MG), and 13 (Structure of the inner walls of the MG) were recoded (Table S5.2). This procedure, at least partly, reduced the problem of low numbers of cases per category and is conservative regarding our hypothesis that male beewolves should show pronounced differences in their gland morphology.

According to the results of an initial CATPCA analysis with the combined dataset (Table S5.2) including all 14 characters of MG and PPG, several changes to the dataset and the analysis parameters were specified according to Linting et al. (2007) and Linting & van der Kooij (2012). First, based on the relatively low Eigenvalues (= variance accounted for) obtained for variables 9 (Structure of the MG) and 13 (Structure of the inner walls of the MG) in the initial CATPCA run (Table S5.3), we decided to exclude these variables from the final analysis (Linting & van der Kooij 2012). Second, to facilitate the interpretation of the resulting CATPCA plot, we also excluded character 11 (Location of the MG), as the initial analysis revealed a strong redundancy of this character with regard to character 10 (Relative size of the MG) (i.e. same vector coordinates for both characters in the CATPCA plot; see Figure 5.4). Despite the somewhat higher Eigenvalue of 11 (Location of the MG, Table S5.3), we rather retained 10 (Relative size of the MG) in the analysis

as we deemed the size of the MG the biologically more relevant trait. Thus, 11 morphological characters were retained in the final CATPCA analysis. Third, while in the initial analysis the level of all variables used for the optimal scaling transformation was nominal, in the final CATPCA run, for characters 2 (Relative size of the PPG), 3 (Branching of the PPG), 10 (Relative size of the MG), and 12 (Branching of the MG) the analysis level was changed to ordinal, based on their transformation plots (i.e. the categories of a variable plotted against the category quantifications assigned to it by the optimal scaling transformation; Linting et al. 2007, Linting & van der Kooil 2012). Moreover, the phylogenetic/ phylogeographic affiliation of the species was included as a supplementary variable in the final CATPCA run. A supplementary variable does not influence the computation of the CATPCA solution, but is rather fitted to the solution afterwards to depict its relation to the other variables in the model (Meulman et al. 2004b).

In certain taxonomic groups of the Philanthinae the MG is missing (see Results), leading to structural zeros due to nested variables in the dataset (e.g. information on MG size and branching missing due to the absence of this gland). The CATPCA offers an elegant way to handle such structural zeros, by giving the option to assign an extra category to missing values during the transformation process, rather than omit them from the analysis (Meulman et al. 2004b, Linting & van der Kooij 2012). This option was applied to MG characters.

**Table S5.3** Eigenvalues of the 14 morphological characters of the head glands of male Philanthinae included in the initial CATPCA run (Eigenvalue initial run) and the Eigenvalues of the 11 characters retained in the final CATPCA run (Eigenvalue final run). The numbering of the characters (No.) corresponds to the list of characters in sections 5.3.4.1 and 5.6.1. Characters shaded in grey were excluded from the final CATPCA run (see section 5.6.2.1).

| No. | Character | Eigenvalue initial run | Eigenvalue final run |
|---|---|---|---|
| **1** | Overall structure of the PPG | 1.242 | 1.087 |
| **2** | Relative size of the PPG | 0.960 | 0.914 |
| **3** | Modifications of PPG morphology | 1.185 | 1.008 |
| **4** | Branching of the PPG | 0.986 | 0.963 |
| **5** | Number of openings of the lower PPG | 1.159 | 1.006 |
| **6** | Structure of the inner walls of the PPG | 0.987 | 0.843 |
| **7** | Type of gland cells associated with the PPG | 1.507 | 1.207 |
| **8** | Presence of the MG | 1.531 | 1.212 |
| **9** | Overall structure of the MG | 0.305 | - |
| **10** | Relative size of the MG | 0.584 | 0.614 |
| **11** | Location of the MG in the head capsule | 0.964 | - |
| **12** | Branching of the MG | 0.480 | 0.459 |
| **13** | Structure of the inner walls of the MG | 0.306 | - |
| **14** | Type of gland cells associated with the MG | 0.944 | 0.920 |

*5.6.2.2 Phylogenetic generalized least squares regressions on gland size and complexity*

We tested for an opposing trend between MG and PPG with regard to their size and complexity by applying phylogenetic generalized least squares regressions based on the molecular phylogeny of Kaltenpoth et al. (2014) in R (Version 3.3.3) (R Core Team 2017) using the package 'ape' (Paradis et al. 2004). As with the CATPCA, *P. albopilosus* (ID 18) was excluded from this analysis, as well as the Nearctic *Philanthus gibbosus* (ID 22), for which the size of the PPG reservoir could not be assessed (Tables S5.1 and S5.2). Moreover, since the molecular phylogeny comprised only one unidentified *Cerceris* species (Kaltenpoth et al. 2014), we used only *C. rybyensis* (ID 2) and *Clypeadon laticinctus* (ID 3, omitting *Cerceris quinquefasciata*, ID 4). We regressed MG and PPG size and complexity, respectively, using phylogenetic independent contrasts by applying the 'gls'-function of the 'ape'-package with the correlation argument 'corBrownian'. The amount of phylogenetic signal in the possible correlations of gland size and complexity was assessed by estimating Pagel's λ (Pagel 1999) using the 'gls'-function with the correlation argument 'corPagel'. Pagel's λ is an estimate for the strength of phylogenetic signal in the data, with values close to 0 indicating no phylogenetic signal, whereas values close to 1 indicate a strong phylogenetic signal (e.g. Münkemüller et al. 2012). For both, size and complexity the software estimated λ-values close to 1. However, possibly due to the comparatively small number of species in our data set (Münkemüller et al. 2012), testing these estimated models against models with λ=0 and λ=1 (created by using the 'gls'-function with the correlation argument 'corPagel' with 'value=0' or 'value=1', respectively), by likelihood ratio $\chi^2$ tests (using the 'anova'-function of 'ape'), revealed that for both, size and complexity, λ was significantly different from 0 (size and complexity: $p < 0.001$ respectively) and from 1 (size: $p = 0.013$, complexity: $p = 0.0065$). This is probably the result of the relatively small number of species under study. As a consequence, the results of the correlation analysis with the estimated λ of 1 have to be interpreted with care.

*5.6.2.3 Hierarchical cluster analysis*

The hierarchical cluster analysis (HCA) was based on 13 of the 14 gland characters. Character 8 (Presence of the MG) was excluded, as in the combined dataset it would have been largely redundant with regard to character 10 (Relative size of the MG, Table S5.2). Fifteen species of the Philanthinae completely lack the MG (see Results) so that the MG characters could not be defined. To assess a possible effect of these structural zeros on the grouping of species, we repeated the analysis with the most important MG characters combined to one, thus avoiding structural zeros. In this compound character, we summarized the information from the four MG

characters 10 (Relative size of MG), 11 (Location of MG within the head capsule), 12 (Branching of MG reservoir), and 14 (Type of gland cells associated with the MG) by assigning a separate category to each of the different combinations of character states in the four original characters (12 different combinations). Yet, we could not include all original MG characters, as this would have resulted in too many categories. We excluded characters 9 (Overall structure of the MG) and 13 (Structure of the inner walls of the MG), based on their comparatively small impact on the clustering of species in the CATPCA (i.e. low Eigenvalues, see section 5.6.2.1 above). The use of a compound character for the MG altered the grouping of single species (result not shown), but did not change the general outcome as compared to the HCA including structural zeros.

*5.6.2.4 Phylogenetic trends: Cophylogenetic analysis*

The cophylogenetic analysis was run with the default cost parameters of Jane 4, using all possible combinations of either edge- or node-based cost models and either randomizing the parasite (i.e. morphology-based) tree (β=-1) or permuting host-parasite associations (100 resamplings, respectively). After having tested several combinations, which did not influence the results, the number of generations was set to 30 and the population size to 300 for all analyses. As the molecular phylogeny of Kaltenpoth et al. (2014) included more species than our morphology-based cluster analysis, we conducted the cophylogenetic analysis using (1) the original molecular phylogeny from Kaltenpoth et al. (2014), (2) the molecular phylogeny including only the 33 species also included in our morphology-based tree, and (3) the molecular phylogeny including only the same 33 species, combined with a morphology-based tree in which we treated all species showing no difference in gland morphology (see Results and Tables S5.1 and S5.2 in section 5.6.1) as a single tip using the possibility in Jane 4 to assign multiple host associations between parasite and host trees.

**5.6.3 Digital photos**

All digital microscope photos (Figure 5.2, Figure S5.2) were optimized for color, contrast, and sharpness using the software Adobe Photoshop Elements (Version 5.0; Microsoft, Redmond, USA).

### 5.6.4 Additional Results: Male gland morphology

#### *5.6.4.1 Additional figure: Gland cell morphology*

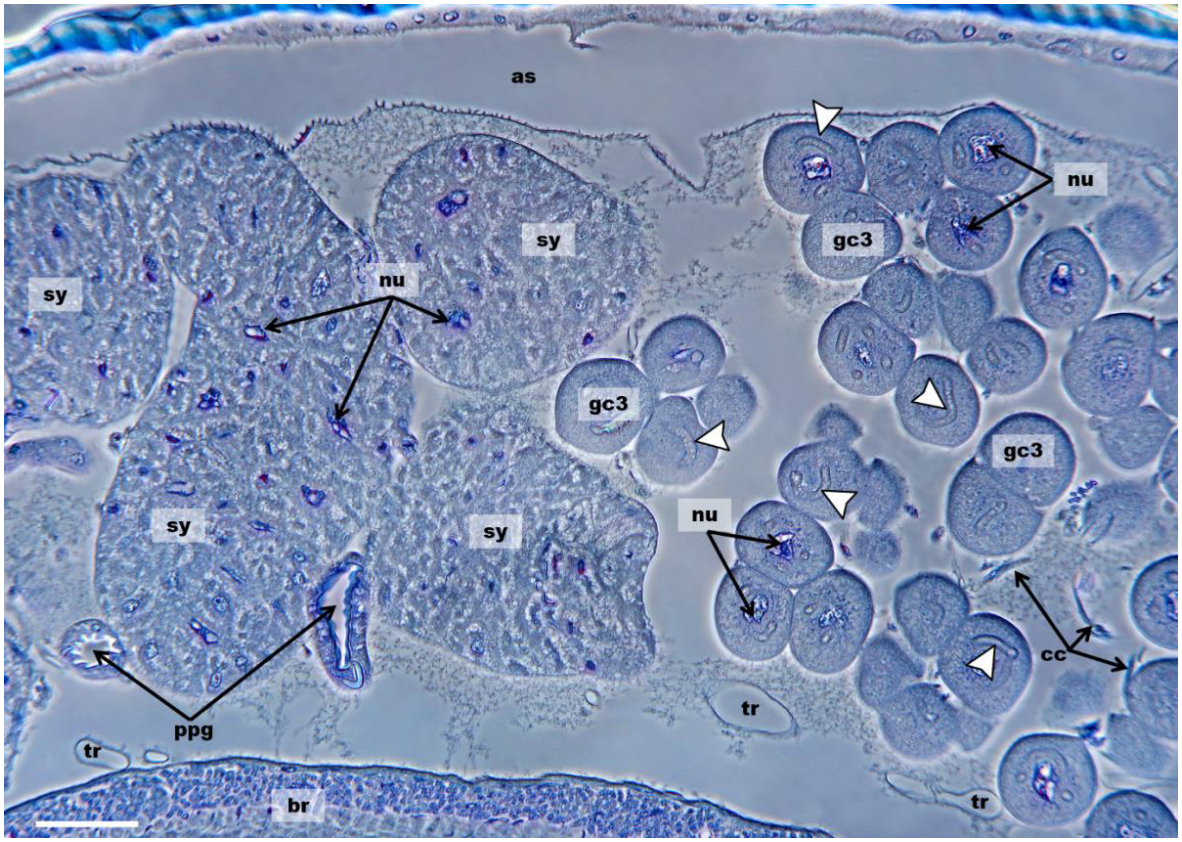


**Figure S5.2** Semithin sagittal section through the head of a male *Philanthus rugosus* showing parts of both, the postpharyngeal gland (PPG) and the mandibular gland (MG), each with associated secretory cells. Multinuclear syncytia surround the fine branches of the PPG reservoir (left side), while the MG is associated with typical NQ-class 3 cells (right side) characterized by end apparatus (white arrow heads) and conducting canals that connect the gland cells with the MG reservoir. Abbreviations: as, air sac; br, brain; cc, conducting canal; gc3, NQ-class 3 gland cells; nu, cell nucleus (with nucleoli); ppg, fine branches of the PPG; sy, multinuclear syncytia; tr, tracheole. Scale bar = 50 µm.

*5.6.4.2 Phylogenetic trends in gland morphology: Hierarchical cluster analysis*

The clustering of species in the hierarchical cluster analysis (see 5.4.3), largely, but not entirely, corresponds to the phylogenetic and phylogeographic relationships within the subfamily (according to Kaltenpoth et al. 2014). In the following, important shifts in in gland morphology are described (Numbers in circles in Figure S5.3). (1) All species lacking the MG and/or possessing the most complex type of gland cells associated with the PPG (i.e. multinuclear syncytia or cell aggregations interspersed with small cells), that is all Nearctic *Philanthus* (including *P. multimaculatus* (ID 24) possessing an MG, and *P. albopilosus* (ID 18) completely lacking secretory cells) as well as the Indian *P. pulcherrimus* (ID 6) and the Palearctic *P. venustus* (ID 8), are separated from those philanthine species possessing both MG and PPG but lacking the small cells interspersed in the syncytia of the PPG. (2) Within the cluster possessing both head glands, members of the tribes Cercerini and Aphilanthopsini (IDs 1-3) as well as the one investigated representative of the genus *Philanthinus* (ID 4) form a separate cluster, as their MGs are either much larger than their PPGs or at least of approximately equal volume, while in the other species in this cluster the MG is smaller than the PPG. (3) The two *Trachypus* species are placed into a separate branch, presumably by a unique combination of traits, including the lack of the lower part of the PPG, the presence of only single NQ-class 3 cells at the MG, and the possession of only the lower part of the MG (note however, that the later only holds true for *T. elongatus* (ID 32), while the structure of the MG of *T. flavidus* could not be determined (Tables S5.1 and S5.2), hence the clustering of *Trachypus* may be interpreted with caution). (4) Within the cluster of Palearctic, Indian, and Afrotropical *Philanthus*, species possessing gland cells associated with both the MG and PPG (IDs 7 and 10-15), are separated from the four species of this cluster possessing only gland cells associated with the MG (IDs 5, 9, 16, 17). (5) The two subspecies of *P. triangulum* (IDs 16 and 17) and the Indian *P.* cf. *basalis* (ID 5) form a separate cluster based on the occurrence of both lower and upper reservoir of the MG in these species. (6) Unlike all other Palearctic/Afrotropical *Philanthus*, *P. histrio* (ID 12) and *P. rugosus* (ID 15) possess only single NQ-class 3 cells and are placed into a separate branch. (7) *P. albopilosus* (ID 18), possessing only a very small PPG and no gland cells, is separated from all other Nearctic *Philanthus*, possessing well developed PPGs. (8) *P. multimaculatus* (ID 24) is placed into an unique branch within the Nearctic *Philanthus* as it is the only species of this cluster possessing an MG. (9) The Palearctic *P. venustus* (ID 8) is placed into an unique branch as the secretory cells of its PPG form aggregations of single cells, rather than multinuclear syncytia.

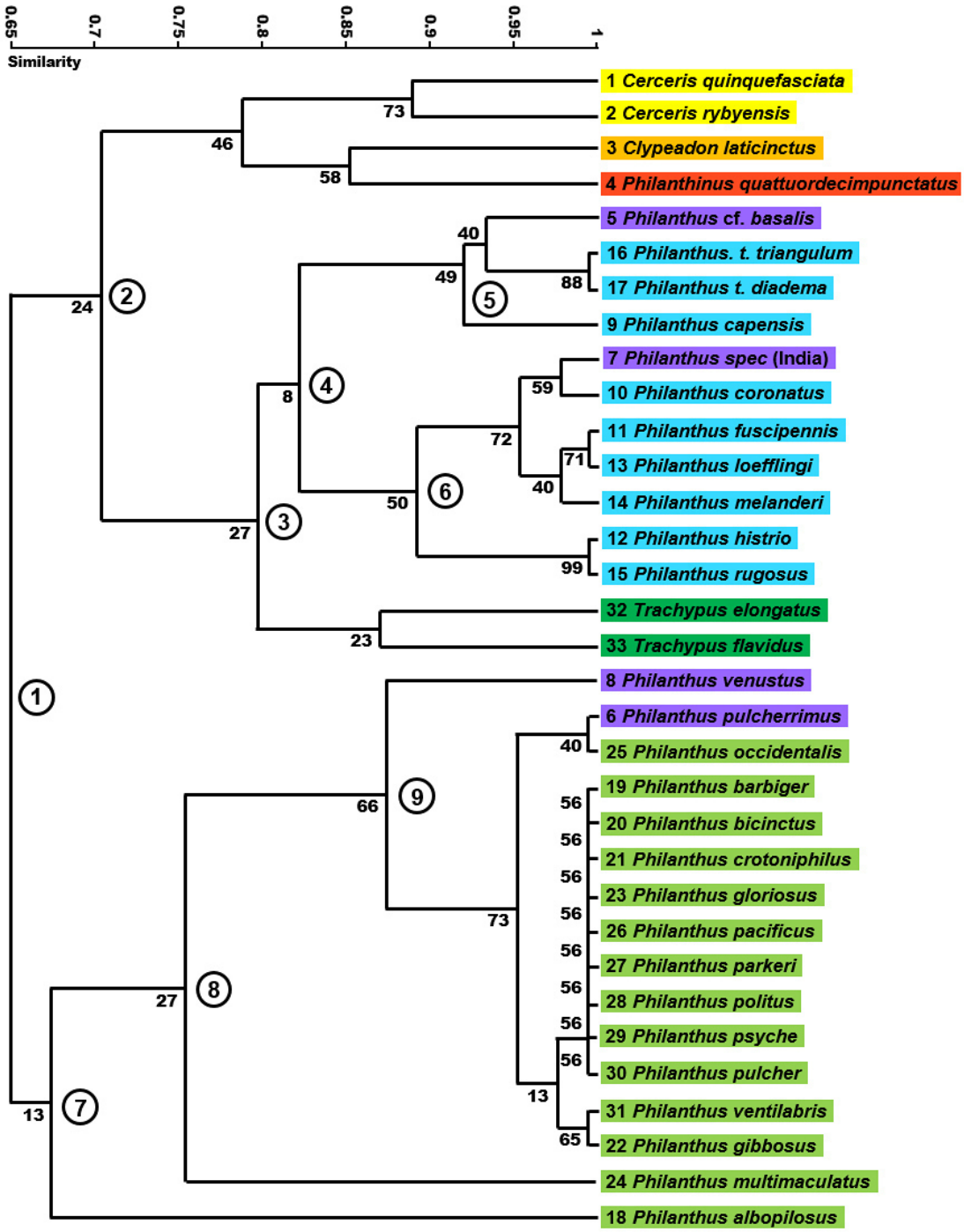


**Figure S5.3** Dendrogram resulting from the hierarchical cluster analysis of head gland morphology of male Philanthinae. Color code: yellow, genus *Cerceris*; orange, genus *Clypeadon*; red, genus *Philanthinus*; purple, Palearctic/Indian *Philanthus*; blue, Palearctic/Afrotropical *Philanthus*; light green, Nearctic *Philanthus*; dark green, genus *Trachypus*. Species IDs correspond to Table 5.1. Numbers in circles indicate important shifts in gland morphology (see text above for details). Not encircled numbers at nodes are bootstrap values. Bray-Curtis was used as the similarity measure; N bootstrap replicates = 10,000.

*5.6.4.3 Additional figures: Ancestral state reconstructions*

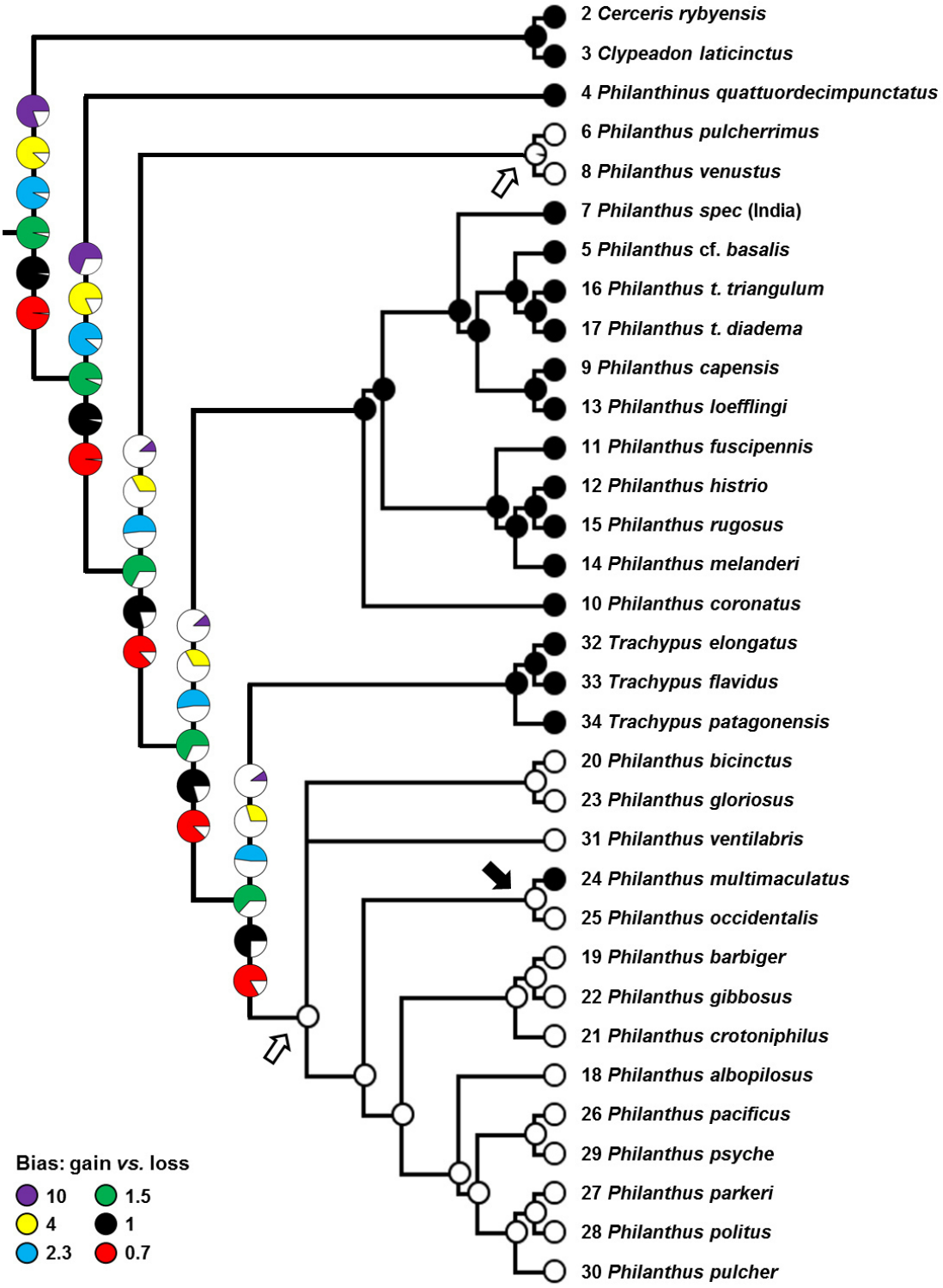


**Figure S5.4** Ancestral state reconstruction for the occurrence of the mandibular gland (MG) of male Philanthinae. The maximum likelihood (ML) reconstruction of presence vs. absence of the MG (character 8, Tables S5.1 and S5.2) was based on the molecular phylogeny of Kaltenpoth et al. (2014). Pie charts at the nodes give the ML probabilities for presence (color) vs. absence (white) of an MG. For relevant nodes results of models with different biases are shown (Color code see key in the figure), for all other nodes the results of the unbiased model are given (i.e. gains and losses equally likely, black). The unbiased model revealed two independent losses (white arrows) and one gain (black arrow) of the MG among the investigated species.

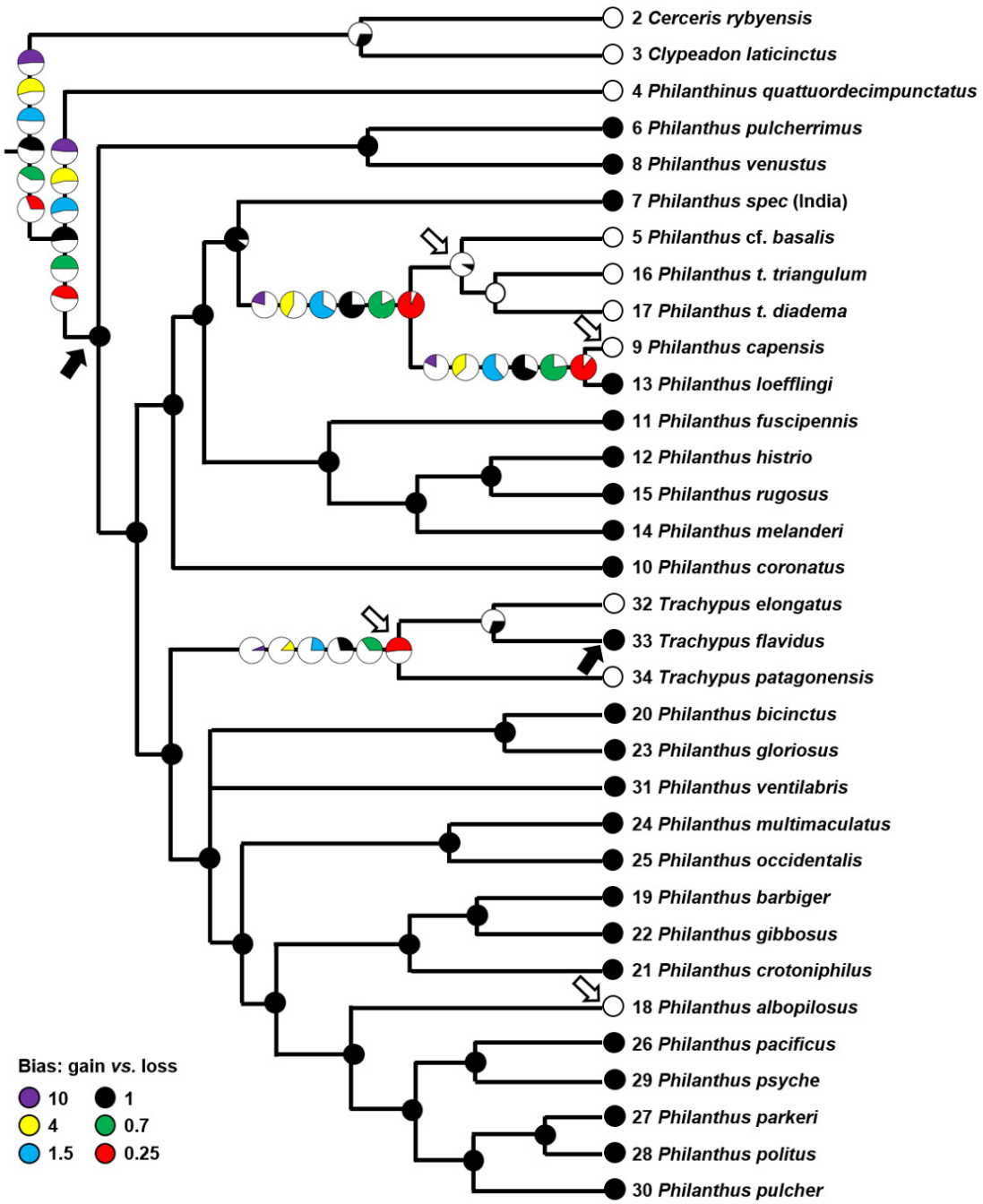


**Figure S5.56** Ancestral state reconstruction for the presence of the presumed secretory cells of the postpharyngeal gland (PPG) in male Philanthinae. The maximum likelihood (ML) reconstruction of presence *vs.* absence of the presumed secretory cells (state 0 *vs*. all other states of character 7, Tables S5.1 and S5.2) was based on the molecular phylogeny of Kaltenpoth et al. (2014). Pie charts at the nodes give the ML probabilities for the presence (color) vs. absence (white) of secretory cells. For relevant nodes results of models with different biases are shown (Color code see key in the figure), for all other nodes the results of the unbiased model are given (i.e. gains and losses equally likely, black). The unbiased model revealed two independent gains (black arrows) and four independent losses (white arrows) of the secretory cells of the PPG among the investigated species.

### 5.6.5 Definition and coding of aggregated characters of head glands of males and females

In order to test whether the interspecific diversity in the morphology of postpharyngeal gland (PPG) and mandibular gland (MG) is higher in male than in female Philanthinae, the characters and character states of both sexes had to be represented in the same coordinate system by a categorical principal components analysis (CATPCA). To accomplish this, we created an aggregate data matrix by combining and recoding 13 characters of female head glands derived from Weiss et al. (2015) and Weiss et al. (*in preparation*) and 14 characters of male head glands derived from the 'combined data' set of this study (Table S5.2, section 5.6.1 above). The resulting aggregated dataset comprises 17 morphological characters of MG and PPG of both sexes (Table S5.2; see also section 5.4.2). Thirteen of these 17 joint characters represent combinations of the equivalent characters of both sexes (or, in the case of character 12, a combination of one male and two female characters). The different character states of the joint character represent combinations of one to four states of the combined original characters. However, four characters could not be combined since they were unique to males (characters 16 and 17, Table S5.4) or to females (characters 14 and 15, Table S5.4) (see section 5.6.6 below for details on the treatment of the resulting structural zeroes due to a missing character or lack of information on the character state). Some characters had been much more finely differentiated in females (Weiss et al. 2015) than in males because in the latter the considerable variation in the same character was often so pronounced that fine-graded variations were not coded. Therefore, for some joint characters different original female character states were equaled to the appropriate state as defined for males.

The following description of the aggregated characters and their states provides the number of the joint character and character state in black, as well as the respective original numbers of the characters and states in males [blue] (see Supplementary Material section 5.6.1 above) and females [red] (Weiss et al. 2015). Hyphens [-] indicate that characters or character states were not defined in the respective original dataset. For more details, see description of male characters in the Supplementary Material section 5.6.1 and Weiss et al. (2015) for females.

**MG:**

**1** [8] [-] *Presence of the MG reservoir*. Whereas in some Philanthinae, males lack the MG reservoir a MG is present in all females. Hence, the presence of the MG was not originally included as a character in the dataset of female head gland morphology.

**(0)** (0) (-) no MG reservoir

**(1)** (1) (-) MG reservoir present

**2** [9] [9] *Structure of the MG reservoir*.

**(0)** (0) (1) only the upper part of the MG reservoir is present

**(1)** (1) (0) only the lower part of the MG reservoir is present

**(2)** (2) (2) both upper and lower parts of the MG reservoir are present

**3** [10] [10] *Relative size of the MG reservoir*. In both sexes we assessed the relative size of the MG as its extension in relation to the head capsule.

**(0)** (-) (0) very small MG (spanning < 25% of head capsule)

**(1)** (0) (1) (2) small MG (spanning approximately 25 - 50% of the head capsule)

**(2)** (1) (-) medium sized MG (spanning approximately 50 - 100% of the head capsule)

**(3)** (2) (-) large MG (spanning 100 - 200% of the head capsule)

**4** [11] [-] *Location of the MG reservoir in the head capsule*. Due to its relatively small size, the MG of female Philanthinae is always located anterior to the brain. Therefore, this character was not originally included in the investigation of female head glands. Here, all females were assigned to category (0). See Supplementary Material section 5.6.1, character 11 for definition of character states.

**(0)** (0) (-) A

**(1)** (1) (-) A + D

**(2)** (2) (-) A + D + B

**(3)** (3) (-) A + D + B + V

**5** [12] [11] *Branching of the MG reservoir*.

**(0)** (0) (0) unbranched: the MG consists of paired sac-like evaginations as reservoirs

**(1)** (1) (1) low: the MG consists of paired reservoirs, each with a few distinct branches

**(2)** (2) (-) high: the MG consists of paired reservoirs, each with many branches

**6** [13] [12] *Structure of the inner walls of the MG reservoir*. The epithelial cells of the MG reservoir can bear cuticular hairs on their inner side, reaching into the lumen of the gland. In females, these hairs generally occur singularly, but in males of some species several hairs can also jointly originate from the top of or laterally from cuticular ridges.

**(0)** (0) (0) unstructured

**(1)** (1) (1) bearing scattered fine hairs

**(2)** (2) (2) bearing many hairs

**7** [14] [13] *Type of gland cells associated with the MG*.

**(0)** (-) (0) the MG reservoir is not associated with gland cells

**(1)** (0) (-) MG-type 1: single gland cells that show end apparatuses, thus resembling NQ-class 3 cells, but lack the canal cells typically connecting NQ-class 3 cells to a reservoir; instead, these cells are tightly arranged around the MG and are directly associated with the wall of the reservoir

**(2)** (1) (1) MG-type 2: single NQ-class 3 gland cells are connected to the MG reservoir via conducting canals

**(3)** (2) (-) MG-type 3: acini, i.e. several NQ-class 3 cells are tightly arranged in cell clusters and jointly connect to the MG reservoir via a bundle of conducting canals

**(4)** (3) (-) MG-type 4: different parts of the MG are associated with MG-type 1 and MG-type 2 cells, respectively

**PPG:**

**8** [1] [1] *Overall structure of the PPG reservoir*.

**(0)** (0) (0) PPG consists of both an upper reservoir originating from the dorsal side of the pharynx and a lower evagination originating ventrally from the pharynx

**(1)** (1) (1) PPG consists of only the upper reservoir

**9** [-] [4] *Number of openings of the upper part of the PPG to the pharynx.* Female Philanthinae have either a single opening or separate openings for the right and left part of the upper PPG reservoir whereas males have invariably separate openings. Hence, this character was not included in the original dataset of males. For the joint dataset, males were assigned to category (1).

**(0)** (-) (0) right and left part of the upper PPG reservoir share one opening to the pharynx

**(1)** (-) (1) both right and left part of the upper PPG each have a separate opening to the pharynx

**10** [5] [8] *Number of openings of the lower part of the PPG to the pharynx.*

**(0)** (0) (0) the lower PPG reservoir has one single opening to the pharynx

**(1)** (1) (1) the lower PPG reservoir has two separate openings to the pharynx

**11** [6] [6] *Structure of the inner walls of the PPG*. The variations in density and distribution of epithelial hairs in females (Weiss et al. 2015) are small compared to the respective variation in males. Thus, here we combined all characters states of the female data set.

**(0)** (0) (0) unstructured

**(1)** (1) (-) cuticular folding

**(2)** (2) (1) (2) (3) (4) hairs

**12** [3] [2] [7] *Modifications of the PPG morphology*. Since the PPG of female Philanthinae is less complex and shows much less interspecific variation, it was not originally recorded as in males (Table S5.2, section 5.6.1). Here, we recoded the two characters that were used to define the shape of the upper and lower PPG of females (Weiss et al. 2015) to match the definitions of PPG 'modules' established for males. We added two new modules that describe the PPG parts of females: **(F)** Unbranched tube-shaped reservoir originating ventrally from the pharynx. **(G)** glove-shaped upper PPG, extending laterally towards the compound eyes in front of the brain and consisting of a common root from which numerous 'fingers' branch off; this part constitutes the typical upper PPG of female Philanthini and is not found in males. See Supplementary Material section 5.6.1, character 3 for definition of character states.

**(0)** (0) (-) A

**(1)** (1) (-) A + D

**(2)** (2) (-) A + E

**(3)** (-) (0)[1] + (1)[2] A + F

**(4)** (3) (-) A + B

**(5)** (4) (-) A + B + D + E

**(6)** (5) (-) A + B + C

**(7)** (6) (-) A + B + C + E

**(8)** (7) (8) (-) A + B + C + D + E

**(9)** (-) (1)[1] (2)[1] (3)[1] G

**(10)** (-) (1)[1] (2)[1] (3)[1] + (0)[2] G + E

[1] Character states of female character (2) 'Shape of the upper PPG' of Weiss et al. (2015)

[2] Character states of female character (7) 'Shape of the lower PPG' of Weiss et al. (2015)

**13** [7] [-] *Type of gland cells associated with the PPG*. In females the PPG has never been found to be associated with secretory cells, hence this character was not originally defined in the morphological investigation of females. In the 'joint dataset', females are assigned to category (0).

**(0)** (0) (-) no gland cells

**(1)** (1) (-) PPG-type 1: several gland cells are tightly associated and form cell aggregations that are directly associated with very fine branches of the PPG (see character 17)

**(2)** (2) (-) PPG-type 2: similar to PPG-type 1, but the cell aggregations are interspersed with small rounded cells (Figure 5.2 D)

**(3)** (3) (-) PPG-type 3: the secretory cells consist of syncytia (i.e. aggregations of cells but no cell membranes visible between cells) that are directly associated with very fine branches of the PPG (see character 17) (Figure 5.2 E)

**(4)** (4) (-) PPG-type 4: similar to PPG-type 3 but the syncytia are interspersed with small rounded cells (similar to those described for PPG-type 2) (Figure 5.2 F)

**14** [-] [5] *Relative lateral extension of the upper PPG of females*. For females, the shape of the PPG required that its relative size was estimated as the lateral extension within the head capsule (which represents, however, the longitudinal axis of the gland) as opposed to males where the longitudinal extension was determined (see character 16).

**(0)** (0) 45 % or less

**(1)** (1) 46 - 50 %

**(2)** (2) 51 - 55 %

**(3)** (3) 56 - 60 %

**(4)** (4) 61 - 65 %

**(5)** (5) 66 - 70 %

**(6)** (6) 71 - 75 %

**(7)** (7) > 75 %

**15** [-] [3] *Number of lobes (per side) of the upper PPG of females*. Again, due to the difference in shape of PPG between the sexes, the degree of branching, i.e. the number of 'fingers' of the glove- or comb-shaped female PPGs, was determined by assessing the average number of 'fingers' on each side of the laterally symmetric upper PPG.

**(0)** (0) 1

**(1)** (1) < 10

**(2)** (2) 10 - 15

**(3)** (3) > 15

**16** [2] [-] *Relative dorsal extension of the upper PPG reservoir of males*.

**(0)** (0) small PPG (spanning approximately 25 - 50% of the head capsule)

**(1)** (1) medium sized PPG (spanning approximately 50 - 100% of the head capsule)

**(2)** (2) large PPG (spanning 100 - 200% of the head capsule, i.e. it reaches around the brain and extends downwards towards the mandible base ventrally to the brain)

**17** [4] [-] *Branching of the PPG reservoir of males*.

**(0)** (0) unbranched

**(1)** (1) some quite voluminous branches originating from the main reservoir of the upper PPG

**(2)** (2) fine branches associated with single gland cell units

**Table S5.4** Aggregated dataset of head gland morphology for both sexes of Philanthinae. Species IDs for males correspond to Table 5.1. The numbering of the characters and the numeric coding of the character states correspond to the description in section 5.6.5 above. (?) character state could not be determined, (-) character not present in this species.

| | | MG | | | | | | | PPG | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Males** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
| 1 | *Cerceris quinquefasciata* | 1 | 0 | 3 | 3 | 2 | 1 | 1 | 1 | 1 | - | 1 | 0 | 0 | - | - | 0 | 0 |
| 2 | *Cerceris rybyensis* | 1 | 0 | 3 | 2 | 1 | 1 | 4 | 1 | 1 | - | 1 | 0 | 0 | - | - | 1 | 0 |
| 3 | *Clypeadon laticinctus* | 1 | 0 | 3 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 0 | - | - | 1 | 0 |
| 4 | *P. quattuordecimpunctatus* | 1 | 0 | 3 | 2 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 5 | 0 | - | - | 1 | 1 |
| 5 | *Philanthus* cf. *basalis* | 1 | 2 | 3 | 1 | 0 | 1 | 3 | 1 | 1 | - | ? | 4 | 0 | - | - | 2 | 1 |
| 6 | *Philanthus pulcherrimus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 6 | 4 | - | - | 2 | 2 |
| 7 | *Philanthus spec* (India) | 1 | 0 | 2 | 1 | 0 | 2 | 3 | 1 | 1 | - | 0 | 6 | 3 | - | - | 2 | 2 |
| 8 | *Philanthus venustus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 2 | 8 | 2 | - | - | 2 | 2 |
| 9 | *Philanthus capensis* | 1 | 0 | 3 | 1 | 1 | 1 | 3 | 1 | 1 | - | 2 | 4 | 0 | - | - | 2 | 1 |
| 10 | *Philanthus coronatus* | 1 | 0 | 2 | 1 | 0 | 1 | 3 | 1 | 1 | - | 0 | 6 | 3 | - | - | 2 | 2 |
| 11 | *Philanthus fuscipennis* | 1 | 0 | 2 | 1 | 1 | 1 | 3 | 1 | 1 | - | ? | 6 | 3 | - | - | 2 | 2 |
| 12 | *Philanthus histrio* | 1 | 0 | 1 | 0 | 0 | 2 | 2 | 1 | 1 | - | 2 | 6 | 3 | - | - | 2 | 2 |
| 13 | *Philanthus loefflingi* | 1 | 0 | 2 | 1 | 1 | 1 | 3 | 1 | 1 | - | 2 | 6 | 3 | - | - | 2 | 2 |
| 14 | *Philanthus melanderi* | 1 | 0 | 2 | 1 | 0 | 1 | 3 | 1 | 1 | - | 2 | 6 | 3 | - | - | 2 | 2 |
| 15 | *Philanthus rugosus* | 1 | 0 | 1 | 0 | 0 | 2 | 2 | 1 | 1 | - | 2 | 6 | 3 | - | - | 2 | 2 |
| 16 | *Philanthus t. triangulum* | 1 | 2 | 3 | 1 | 0 | 2 | 3 | 1 | 1 | - | 1 | 4 | 0 | - | - | 2 | 1 |
| 17 | *Philanthus t. diadema* | 1 | 2 | 3 | 1 | 0 | 2 | 3 | 1 | 1 | - | 1 | 4 | 0 | - | - | 2 | 1 |
| 19 | *Philanthus barbiger* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 20 | *Philanthus bicinctus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 21 | *Philanthus crotoniphilus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 22 | *Philanthus gibbosus* | 0 | - | - | - | - | - | - | ? | 1 | ? | 0 | ? | 4 | - | - | ? | ? |
| 23 | *Philanthus gloriosus* | 0 | - | - | - | - | - | - | 0 | ? | ? | 0 | 8 | 4 | - | - | 2 | 2 |
| 24 | *Philanthus multimaculatus* | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 25 | *Philanthus occidentalis* | 0 | - | - | - | - | - | - | ? | ? | ? | 0 | ? | 4 | - | - | 2 | 2 |
| 26 | *Philanthus pacificus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 27 | *Philanthus parkeri* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 28 | *Philanthus politus* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 29 | *Philanthus psyche* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 30 | *Philanthus pulcher* | 0 | - | - | - | - | - | - | 0 | 1 | 0 | 0 | 8 | 4 | - | - | 2 | 2 |
| 31 | *Philanthus ventilabris* | 0 | - | - | - | - | - | - | 0 | 1 | ? | 0 | ? | 4 | - | - | 2 | 1 |
| 32 | *Trachypus elongatus* | 1 | 1 | 2 | 0 | 0 | 1 | 2 | 1 | 1 | - | 0 | 4 | 0 | - | - | 2 | 1 |
| 33 | *Trachypus flavidus* | 1 | ? | 1 | 0 | ? | 0 | 2 | 1 | ? | - | 0 | 6 | 1 | - | - | 2 | 2 |

**Table S5.4** *continued*

| | | MG | | | | | | | PPG | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Females** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
| 34 | *Cerceris arenaria* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 2 | 3 | 0 | 3 | 0 | - | - |
| 35 | *Cerceris quinquefasciata* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 2 | 3 | 0 | 0 | 0 | - | - |
| 36 | *Cerceris rybyensis* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 3 | 0 | ? | 0 | - | - |
| 37 | *Cerceris sabulosa* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 3 | 3 | 0 | 1 | 0 | - | - |
| 38 | *Aphilanthops frigidus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3 | 0 | 4 | 0 | - | - |
| 39 | *Clypeadon laticinctus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 3 | 0 | 2 | 0 | - | - |
| 40 | *P. quattuordecimpunctatus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 4 | 1 | - | - |
| 41 | *Philanthus venustus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 10 | 0 | 4 | 2 | - | - |
| 42 | *Philanthus capensis* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 10 | 0 | 5 | 2 | - | - |
| 43 | *Philanthus coronatus* | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | 7 | 2 | - | - |
| 44 | *Philanthus loefflingi* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 10 | 0 | 6 | 2 | - | - |
| 45 | *Philanthus melanderi* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | 3 | 2 | - | - |
| 46 | *Philanthus rugosus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 10 | 0 | 4 | 2 | - | - |
| 47 | *Philanthus t. triangulum* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | ? | ? | - | - |
| 48 | *Philanthus t. diadema* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | 5 | 2 | - | - |
| 49 | *Philanthus albopilosus* | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | 5 | 2 | - | - |
| 50 | *Philanthus barbiger* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 10 | 0 | 5 | 2 | - | - |
| 51 | *Philanthus bicinctus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | ? | 10 | 0 | ? | 3 | - | - |
| 52 | *Philanthus crabroniformis* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | ? | ? | ? | ? | 0 | ? | ? | - | - |
| 53 | *Philanthus gibbosus* | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 10 | 0 | 1 | 1 | - | - |
| 54 | *Philanthus multimaculatus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | ? | 2 | - | - |
| 55 | *Philanthus psyche* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 0 | 2 | 10 | 0 | ? | 3 | - | - |
| 56 | *Philanthus pulcher* | 1 | 0 | ? | 0 | ? | 0 | 0 | 0 | 1 | 0 | 2 | ? | 0 | ? | ? | - | - |
| 57 | *Philanthus ventilabris* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 10 | 0 | 4 | 2 | - | - |
| 58 | *Trachypus boharti* | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 1 | 1 | - | 2 | 9 | 0 | 7 | 2 | - | - |
| 59 | *Trachypus elongatus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 10 | 0 | 6 | 2 | - | - |
| 60 | *Trachypus flavidus* | 1 | ? | ? | 0 | ? | ? | 2 | 1 | 1 | - | 2 | 9 | 0 | 6 | 2 | - | - |
| 61 | *Trachypus patagonensis* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | ? | ? | ? | ? | 0 | ? | ? | - | - |

### 5.6.6 Aggregated categorical principal components analysis of males and females

The aggregated dataset (Table S5.4) was subjected to a categorical principal components analysis as described for the data of males (section 5.3.4.3 and Supplementary Material section 5.6.2.1) to represent the head gland characters for males and females in the same coordinate system. Based on the results obtained from the initial CATPCA run including all 17 aggregated characters, the following adjustments of the analysis parameters were made: First, based on their relatively low Eigenvalues (= variance accounted for) in the initial CATPCA run, we decided to exclude the three characters (2) 'Structure of the MG', (9) 'Number of openings of the upper PPG to the pharynx', and (10) 'Number of openings of the lower PPG to the pharynx' from the final analysis (Table S5.5). Second, to facilitate the interpretation of the resulting CATPCA plot, we also excluded character (4) 'Location of the MG in the head capsule', as the initial analysis revealed a strong redundancy of this character with regard to three other characters (i.e. similar vector coordinates of the characters in the CATPCA plot), namely (3) 'Relative size of the MG', (6) ' Structure of the inner walls of the MG', and (7) 'Type of gland cells associated with the MG'. The final CATPCA thus included 13 morphological characters. Third, while the initial analysis level of all variables, i.e. the scaling level used for the optimal scaling transformation, was changed from nominal to ordinal in the final CATPCA run for six characters (Table S5.5).

Two of the characters that were only defined for one sex included measures of PPG size, i.e. the lateral extension of the females' PPG assessed by the number of semithin sagittal sections of the head capsule containing structures assigned to the PPG reservoir (Character 14) and the dorsal extension of the males' PPG relative to the head capsule (Character 16), respectively. Although both represent PPG size in relation to the head capsule, we did not combine them to a joint character since the approaches to measure lateral and dorsal extension of the differently structured PPGs females of and males, respectively, were too different (Weiss et al. 2015, this study). Yet, to exclude at least major effects of these separated characters on the grouping of species in the CATPCA, we tentatively combined the two size characters by including the female size categories into the more grossly defined male categories (compare descriptions of characters 14 and 16 in Supplementary Material section 5.6.5 above). This did not change the results as compared to the analysis including separated characters for male and female PPG size. Thus, we decided to retain the separated characters in the CATPCA.

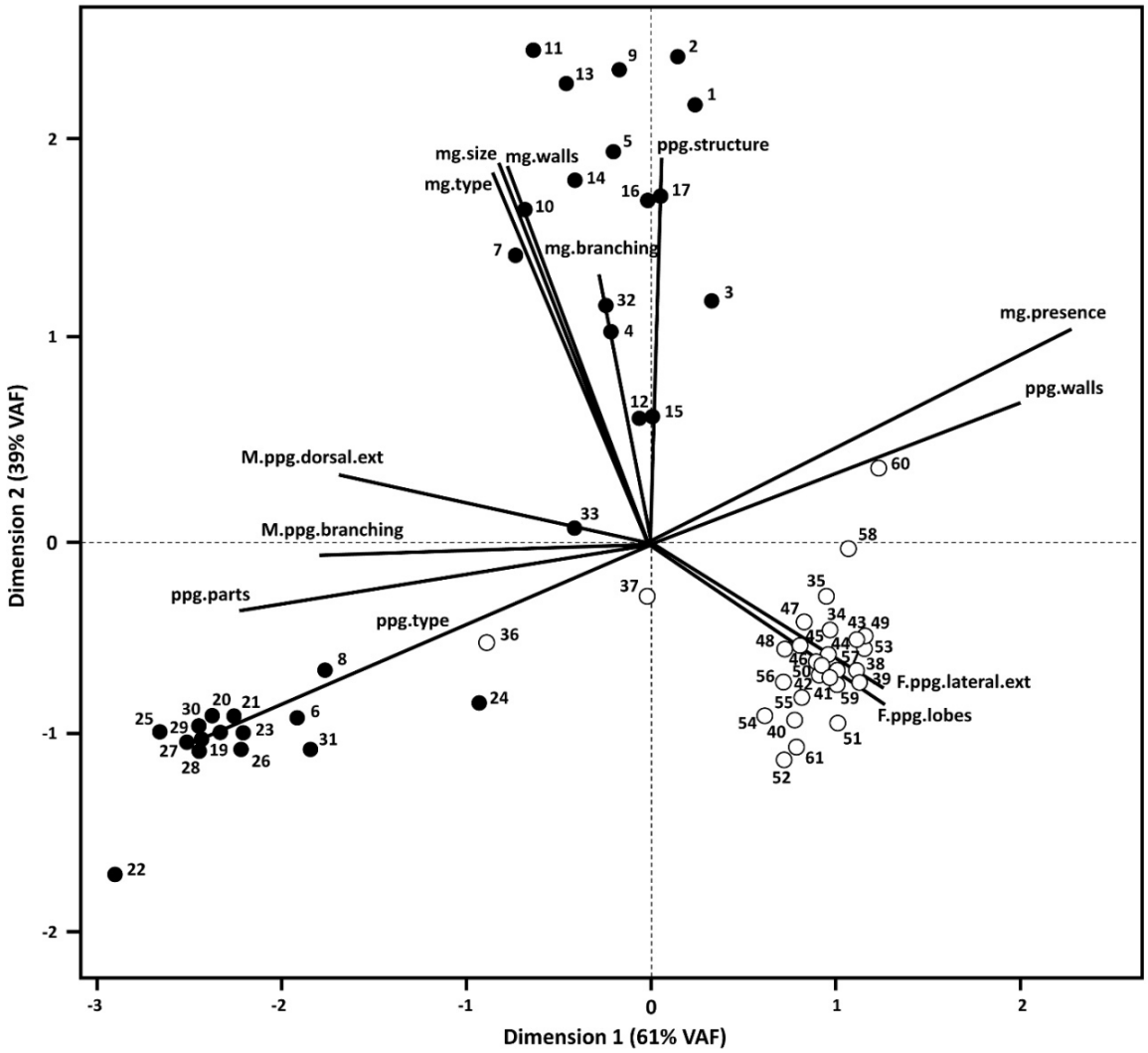


**Figure S5.6** First two dimensions (VAF: percent of variance accounted for) of the categorical principal components analysis (CATPCA) based on the aggregate matrix of morphological characters of the head glands of male (filled circles; IDs correspond to Table 5.1) and female (open circles) Philanthinae (supported by the maximum possible total Cronbach's α of 1, Heiser & Meulman 1994). While most female data points are closely clustered, the data points for males are much more scattered and show two main aggregations similar to the CATPCA including only males (see Figure 5.4). Vectors: component loadings of morphological characters. Abbreviations of morphological characters: (mg.branching) branching of the MG, (mg.presence) presence of the MG, (mg.size) size of the MG relative to the head capsule, (mg.type) type of gland cells associated with the MG, (mg.walls) structure of the inner walls of the MG, (ppg.parts) modifications of PPG morphology, (ppg.structure) overall structure of the PPG, (ppg.type) type of gland cells associated with the PPG, (ppg.wall) structure of the inner walls of the PPG, (F.ppg.lateral.ext) lateral extension of the upper PPG relative to the head capsule in females, (F.ppg.lobes) number of lobes of the upper PPG in females, (M.ppg.branching) branching of the PPG reservoir in males (M.ppg.dorsal.ext), dorsal extension of the upper PPG relative to the head capsule in males.

**Table S5.5** Eigenvalues of the 17 aggregated morphological characters of head gland morphology of male and female Philanthinae included in the initial CATPCA run (Eigenvalue initial run) and the Eigenvalues of the 13 characters retained in the final CATPCA run (Eigenvalue final run). The numbering of the characters (No.) corresponds to the list of characters in section 5.6.5 above. Characters shaded in grey were excluded from the final CATPCA run. The optimal scaling transformation (Level) was changed to ordinal (o) in the final CATPCA run, while it was left nominal (n) in the remaining seven characters.

| No. | Character | Eigenvalue initial run | Eigenvalue final run | Level |
|---|---|---|---|---|
| 1 | Presence of the MG | 1.622 | 1.613 | n |
| 2 | Structure of the MG | 0.204 | - | - |
| 3 | Relative size of the MG | 1.067 | 1.057 | o |
| 4 | Location of the MG in the head capsule | 1.055 | - | - |
| 5 | Branching of the MG | 0.475 | 0.483 | o |
| 6 | Structure of the inner walls of the MG | 1.047 | 1.078 | n |
| 7 | Type of gland cells associated with the MG | 1.112 | 1.079 | n |
| 8 | Overall structure of the PPG | 0.916 | 0.940 | n |
| 9 | Number of openings of the upper PPG to the pharynx | 0.310 | - | - |
| 10 | Number of openings of the lower PPG to the pharynx | 0.336 | - | - |
| 11 | Structure of the inner walls of the PPG | 1.211 | 1.158 | n |
| 12 | Modifications of the PPG morphology | 1.367 | 1.321 | n |
| 13 | Type of gland cells associated with the PPG | 1.868 | 1.738 | n |
| 14 | Relative lateral extension of the upper PPG in females | 0.413 | 0.454 | o |
| 15 | Number of lobes of the upper PPG in females | 0.469 | 0.533 | o |
| 16 | Relative dorsal extension of the upper PPG in males | 0.849 | 0.752 | o |
| 17 | Branching of the PPG reservoir in males | 0.923 | 0.829 | o |

### 5.6.7 Shannon diversity indices for male and female gland morphology

To compare the diversity of gland morphology between the sexes the aggregated dataset was reduced to those characters that were shared by males and females. Therefore, two characters that were only defined in males and two characters that were only defined in females had to be omitted. Following a method described by Tesfaye et al. (1991), Shannon diversity indices of the resulting nine characters for males and females were calculated for each joint character for males and females separately, according to the formula:

$$H = -\sum_{i} \frac{n_i}{n} ln \frac{n_i}{n}$$

With i being the number of categories of a given character, n being the total number of species for which this characters could be assessed, and $n_i$ being the number of species in a given category. The resulting nine indices for both sexes were compared using a Wilcoxon paired test in PAST (Version 2.08b) (Hammer et al. 2001).

## 5.7 References


Adam H, Czihak G: Arbeitsmethoden der makroskopischen und mikroskopischen Anatomie. 1st ed. Stuttgart: Gustav Fischer Verlag, 1964.

Ågren L: Mandibular gland morphology of *Cerceris rybyensis* (L) (Hymenoptera: Philanthidae). *Zoon* 1977, 5(2):91-95.

Alcock J: Male mating strategies of some philanthine wasps (Hymenoptera: Sphecidae). *Journal of the Kansas Entomological Society* 1975, 48(4):532-545.

Alexander BA: A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 1992, 17(2):91-108.

Amundsen T: Why are female birds ornamented? *Trends in Ecology & Evolution* 2000, 15(4):149-155.

Arnegard ME, McIntyre PB, Harmon LJ, Zelditch ML, Crampton WGR, Davis JK, Sullivan JP, Lavoue S, Hopkins CD: Sexual signal evolution outpaces ecological divergence during electric fish species radiation. *The American Naturalist* 2010, 176(3):335-356.

Ayasse M, Paxton R, Tengö J: Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology* 2001, 46(1):31-78.

Bacquet PMB, Brattström O, Wang HL, Allen CE, Löfstedt C, Brakefield PM, Nieberding CM: Selection on male sex pheromone composition contributes to butterfly reproductive isolation. *Proceedings of the Royal Society B: Biological Sciences* 2015, 282(1804).

Billen J: Diversity and morphology of exocrine glands in ants. *Proceedings XIX Simpósio Mirmecologia, Ouro Preto, Brasil* 2009: 17-21.

Billen J: Exocrine glands and their key function in the communication system of social insects. *Formosan Entomology* 2011, 31:75-84.

Billen J, Bauweleers E, Hashim R, Ito F. Survey of the exocrine system in *Protanilla wallacei* (Hymenoptera, Formicidae). *Arthropod Structure & Development* 2013, 42(3):173-183.

Billen J, Mandonx T, Hashim R, Ito F: Exocrine glands of the ant *Myrmoteras iriodum*. *Entomological Science* 2015, 18(2):167-173.

Blum MS: Alarm pheromones. *Annual Review of Entomology* 1969, 14:57-80.

Bohart RM, Menke AS: Sphecid wasps of the world: a generic revision. 1st ed. Ithaca: University of California Press, 1976.

Boonen S, Eelen D, Børgesen L, Billen J: Functional morphology of the mandibular gland of queens of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 2013, 94(4):373-381.

Cane JH, Michener CD: Chemistry and function of mandibular gland products of bees of the genus *Exoneura* (Hymenoptera, Anthophoridae). *Journal of Chemical Ecology* 1983, 9: 1525-1531.

Cane JH, Gerdin S, Wife G: Mandibular gland secretions of solitary bees (Hymenoptera, Apoidea) - Potential for nest cell disinfection. *Journal of the Kansas Entomological Society* 1983, 56(2):199-204.

Cardona A, Saalfeld S, Schindelin J, Arganda-Carreras I, Preibisch S, Longair M, Tomancak P, Hartenstein V, Douglas RJ: TrakEM2 software for neural circuit reconstruction. *PLoS One* 2012, 7(6).

Chadha M, Eisner T, Monro A, Meinwald J: Defence mechanisms of arthropods - VII: citronellal and citral in the mandibular gland secretion of the ant *Acanthomyops claviger* (Roger). *Journal of Insect Physiology* 1962, 8(2):175-179.

Clarke S, Dani F, Jones G, Morgan E, Schmidt J: (Z)-3-hexenyl (R)-3-hydroxybutanoate: a male specific compound in three North American decorator wasps *Eucerceris rubripes*, *E. conata* and *E. tricolor*. *Journal of Chemical Ecology* 2001, 27(7):1437-1447.

Conow C, Fielder D, Ovadia Y, Libeskind-Hadas R: Jane: a new tool for the cophylogeny reconstruction problem. *Algorithms for Molecular Biology* 2010, 5.

Cornwallis CK, Uller T: Towards an evolutionary ecology of sexual traits. *Trends in Ecology & Evolution*. 2010, 25(3):145-152.

Cruz-Landim C: Estudo comparativo de algumas glândulas das abelhas (Hymenoptera, Apoidea) e respectivas implicacoes evolutivas. *Arquivos de Zoologia, Sao Paulo* 1967, 15(3):177-290.

Cruz-Landim C, Costa R: Structure and function of the hypopharyngeal glands of Hymenoptera: a comparative approach. *Journal of Comparative Biology*. 1998, 3(2):151-163.

Cruz-Landim C, Reginato RD: Exocrine glands of *Schwarziana quadripunctata* (Hymenoptera, Apinae, Meliponini). *Brazilian Journal of Biology* 2001, 61(3):497-505.

Cruz-Landim C, Abdalla FC, Gracioli-Vitti LF: Morphological and functional aspects of volatile-producing glands in bees (Hymenoptera: Apidae). *Insect Science* 2005, 12(6):467-480.

Darwin C: On the origin of species by means of natural selection. 1st ed. London: John Murray, 1859.

Darwin C: The descent of man and selection in relation to sex. 1st ed. London: John Murray, 1871.

Debevec AH, Cardinal S, Danforth BN: Identifying the sister group to the bees: a molecular phylogeny of Aculeata with an emphasis on the superfamily Apoidea. *Zoologica Scripta* 2012, 41(5):527-535.

do Amaral JB, Caetano FH: The hypopharyngeal gland of leaf-cutting ants (*Atta sexdens rubropilosa*) (Hymenoptera: Formicidae). *Sociobiology* 2005, 46(3):515-524.

do Amaral JB, Machado-Santelli G: Salivary system in leaf-cutting ants (*Atta sexdens rubropilosa* Forel, 1908) castes: A confocal study. *Micron* 2008, 39(8):1222-1227.

Droney DC, Hock MB: Male sexual signals and female choice in *Drosophila grimshawi* (Diptera: Drosophilidae). *Journal of Insect Physiology* 1998, 11(1):59-71.

Duffield RM, Shamim M, Wheeler JW, Menke AS: Alkylpyrazines in the mandibular gland secretions of *Ammophila* wasps (Hymenoptera: Sphecidae). *Comparative Biochemistry and Physiology B* 1981, 70(2):317-318.

Eelen D, Borgesen L, Billen J: Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 2006, 87:101–11.

Elias DO, Hebets EA, Hoy RR: Female preference for complex/novel signals in a spider. *Behavioral Ecology* 2006, 17(5):765-771.

Emlen DJ, Marangelo J, Ball B, Cunningham CW: Diversity in the weapons of sexual selection: horn evolution in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae). *Evolution* 2005, 59(5):1060-1084.

Evans HE: A review of nesting behavior of digger wasps of the genus *Aphilanthops*, with special attention to the mechanism of prey carriage. *Behaviour* 1962, 19:239-260.

Evans HE: Observations on the nesting behavior of wasps of the tribe Cercerini. *Journal of the Kansas Entomological Society* 1971, 44(4):500-523.

Evans HE: Observations on the biology of *Cerceris mimica* Cresson (Hymenoptera: Sphecidae: Philanthinae). *Journal of the Kansas Entomological Society* 2000, 73(4):220-224.

Evans HE, O'Neill KM: Male territorial behavior in four species of the tribe Cercerini (Sphecidae: Philanthinae). *Journal of the New York Entomological Society* 1985, 93(3):1033-1040

Evans HE, O'Neill KM: The natural history and behavior of North American beewolves. 1st ed. Ithaca: Cornell University Press, 1988.

Fortunato A, Turillazzi S, Delfino G: Ectal mandibular gland in *Polistes dominulus* (Christ) (Hymenoptera, Vespidae): Ultrastructural modifications over the secretory cycle. *Journal of Morphology* 2000, 244(1):45-55.

Foster SP, Johnson CP: Signal honesty through differential quantity in the female-produced sex pheromone of the moth *Heliothis virescens*. *Journal of Chemical Ecology* 2011, 37(7):717-723.

Galvani GL, Settembrini BP: Comparative morphology of the head glands in species of Protepeolini and Emphorini (Hymenoptera: Apidae). *Apidologie* 2013, 44(4):367-381.

Garamszegi LZ, Eens M, Erritzøe J, Møller AP: Sexually size dimorphic brains and song complexity in passerine birds. *Behavioral Ecology* 2005, 16(2):335-345.

Goettler W, Strohm E: Mandibular glands of male European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2008, 37(5):363-371.

Grasso D, Romani R, Castracani C, Visicchio R, Mori A, Isidoro N, Le Moli F: Mandible associated glands in queens of the slave-making ant *Polyergus rufescens* (Hymenoptera, Formicidae). *Insectes Sociaux* 2004, 51(1):74-80.

Gwynne DT: Male territoriality in bumblebee wolf, *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae) - Observations on behavior of individual males. *Zeitschrift für Tierpsychology* 1978, 47(1):89-103.

Hammer Ø, Harper DAT, Ryan PD: PAST: Paleontological statistics software package for education and data analysis. *Paleontologica Electronica* 2001, 4.

Haynes KF, Yeargan KV: Exploitation of intraspecific communication systems: Illicit signalers and receivers. *Annals of the Entomological Society of America* 1999, 92(6):960-970.

Hefetz A: Function of secretion of mandibular gland of male in territorial behavior of *Xylocopa sulcatipes* (Hymenoptera, Anthophoridae). *Journal of Chemical Ecology* 1983, 9(7):923-931.

Hefetz A, Errard C, Chambris A, LeNegrate A: Postpharyngeal gland secretion as a modifier of aggressive behavior in the myrmicine ant *Manica rubida*. *Journal Insect Behavior* 1996, 9:709-717.

Heiser W, Meulman J: Homogeneity analysis: exploring the distribution of variables and their nonlinear relationships. In: Greenacre JB, Blasius J, editors. Correspondence analysis in the social sciences: recent developments and applications. 1st ed. New York: Academic Press, 1994. p. 179-209.

Herzner G, Strohm E: Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007, 17(2):R46-R47.

Herzner G, Strohm E: Food wrapping by females of the European Beewolf, *Philanthus triangulum*, retards water loss of larval provisions. *Physiological Entomology* 2008, 33(2):101-109.

Herzner G, Schmitt T, Linsenmair KE, Strohm E: Prey recognition by females of the European beewolf and its potential for a sensory trap. *Animal Behaviour* 2005, 70(6):1411-1418.

Herzner G, Schmitt T, Heckel F, Schreier P, Strohm E: Brothers smell similar: Variation in the sex pheromone of male European Beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implications for inbreeding avoidance. *Biological Journal of the Linnean Society* 2006, 89(3):433-442.

Herzner G, Goettler W, Kroiss J, Purea A, Webb AG, Jakob PM, Roessler W, Strohm E: Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 2007a, 36(2):123-133.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007b, 33(4):849-859.

Herzner G, Engl T, Strohm E: Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011a, 82(2):321-328.

Herzner G, Ruther J, Goller S, Schulz S, Goettler W, Strohm E: Structure, chemical composition and putative function of the postpharyngeal gland of the emerald cockroach wasp, *Ampulex compressa* (Hymenoptera, Ampulicidae). *Zoology* 2011b, 114(1):36-45.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. *PLoS One* 2013, 8.

Higashi M, Takimoto G, Yamamura N: Sympatric speciation by sexual selection. *Nature* 1999, 402(6761):523-526.

Holland B, Rice WR: Perspective: Chase-away sexual selection: antagonistic seduction versus resistance. *Evolution* 1998, 52(1):1-7.

Holt B, Lessard JP, Borregaard MK, Fritz SA, Araujo MB, Dimitrov D, Fabre PH, Graham CH, Graves GR, Jønsson KA, Nogués-Bravo D, Wang Z, Whittaker RJ, Fjeldså J, Rahbek C: An update of Wallace's zoogeographic regions of the world. *Science* 2013, 339(6115):74-78.

Hosken DJ, House CM: Sexual selection. *Current Biology* 2011, 21(2):R62-R65.

Hosken DJ, Stockley P: Sexual selection and genital evolution. *Trends in Ecology & Evolution* 2004, 19(2):87-93.

Hughes WOH, Howse PE, Vilela EF, Goulson D: The response of grass-cutting ants to natural and synthetic versions of their alarm pheromone. *Physiological Entomology* 2001, 26(2):165-172.

Hughes WOH, Howse PE, Goulson D: Mandibular gland chemistry of grass-cutting ants: species, caste, and colony variation. *Journal of Chemical Ecology* 2001, 27(1):109-124.

Huxley JS: Darwin's theory of sexual selection and the data subsumed by it, in the light of recent research. *The American Naturalist* 1938, 72(742):416-433.

Johansson BG, Jones TM: The role of chemical communication in mate choice. *Biological Reviews* 2007, 82(2):265-289.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(17):6359-6364.

Kimball RT, Braun EL, Ligon JD, Lucchini V, Randi E: A molecular phylogeny of the peacock-pheasants (Galliformes: *Polyplectron spp.*) indicates loss and reduction of ornamental traits and display behaviours. *Biological Journal of the Linnean Society* 2001, 73(2):187-198.

Kokko H, Brooks R, McNamara JM, Houston AI: The sexual selection continuum. *Proceedings of the Royal Society B: Biological Sciences* 2002, 269(1498):1331-1340.

Kokko H, Brooks R, Jennions MD, Morley J: The evolution of mate choice and mating biases. *Proceedings of the Royal Society: Biological Sciences* 2003, 270(1515):653-664.

Kopp A, True JR: Evolution of male sexual characters in the Oriental *Drosophila melanogaster* species group. *Evolution & Development* 2002, 4(4):278-291.

Kraaijeveld K, Kraaijeveld-Smit FJL, Maan ME: Sexual selection and speciation: the comparative evidence revisited. *Biological Reviews* 2011, 86(2):367-377.

Kroiss J, Schmitt T, Schreier P, Strohm E, Herzner G: A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum. Journal of Chemical Ecology* 2006, 32(12):2763-2776.

Kroiss J, Lechner K, Strohm E: Male territoriality and mating system in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae): evidence for a "hotspot" lek polygyny. *Journal of Ethology* 2010, 28(2):295-304.

Lalor PF, Hughes WHO: Alarm behaviour in *Eciton* army ants. *Physiological Entomology* 2011, 36(1):1-7.

Lande R: Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution* 1980, 34(2):292-305.

Lenoir A, Fresneau D, Errard C, Hefetz A: Individuality and colonial identity in ants: The emergence of the social representation concept. In: Information processing in social insects. Basel: Birkhäuser Verlag, 1999. p. 219-237.

Linting M, van der Kooij A: Nonlinear Principal Components Analysis with CATPCA: A Tutorial. *Journal of Personality Assessment* 2012, 94:12-25.

Linting M, Meulman JJ, Groenen PJF, van der Kooij AJ: Nonlinear principal components analysis: introduction and application. *Psychological Methods* 2007, 12:336-358.

Maan ME, Seehausen O: Ecology, sexual selection and speciation. *Ecology Letters* 2011, 14(6):591-602.

Maddison WP, Maddison DR: 2015. Mesquite: A modular system for evolutionary analysis, Version 3.04. http://mesquiteproject.org. Accessed 13 Jan 2015

Mahr K, Evans C, Thonhauser K, Griggio M, Hoi H: Multiple ornaments - multiple signaling functions? The importance of song and UV plumage coloration in female superb fairy-wrens (*Malurus cyaneus*). *Frontiers in Ecology and Evolution* 2016, 4(43)

McDaniel CA, Howard RW, Oneill KM, Schmidt JO: Chemistry of male mandibular gland secretions of *Philanthus basilaris* Cresson and *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1987, 13(2):227-235.

McDaniel CA, Schmidt JO, Howard RW: Mandibular gland secretions of the male beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1992, 18(1):27-37.

Melo GAR: Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on the crabronid wasps. *Scientific Papers Natural History Museum the University of Kansas* 1999(14):1-55.

Meulman J, Heiser WJ, SPSS: SPSS Categories 13.0. Chicago: SPSS, 2004a.

Meulman J, van der Kooij A, Heiser WJ: Principal components analysis with nonlinear optimal scaling transformations for ordinal and nominal data. In: Kaplan D, editor. Handbook on quantitative methodology for the social sciences. London: Sage, 2004b. p. 49-70.

Meyer A: The evolution of sexually selected traits in male swordtail fishes (*Xiphophorus*: Poeciliidae). *Heredity* 1997, 79:329-337.

Meyer A, Morrissey JM, Schartl M: Recurrent origin of a sexually selected trait in *Xiphophorus* fishes inferred from a molecular phylogeny. *Nature* 1994, 368(6471):539-542.

Mikheyev AS: Male accessory gland size and the evolutionary transition from single to multiple mating in the fungus-gardening ants. *Insect Science* 2004, 4.

Møller AP, Szép T: Rapid evolutionary change in a secondary sexual character linked to climatic change. *J Evolution Biology* 2005, 18(2):481-495.

Morris M: Further examination of female preference for vertical bars in swordtails: preference for 'no bars' in a species without bars. *Journal of Fish Biology* 1998, 53:56-63.

Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, Schiffers K, Thuiller W: How to measure and test phylogenetic signal. *Methods in Ecology and Evolution* 2012, 3(4):743-756.

Niehuis O, Buellesbach J, Gibson JD, Pothmann D, Hanner C, Mutti NS, Judson AK, Gadau J, Ruther J, Schmitt T: Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* 2013, 494(7437):345-348.

Noirot C, Quennedey A: Fine-structure of insect epidermal glands. *Annual Review of Entomology* 1974, 19:61-80.

Omland KE, Lanyon SM: Reconstructing plumage evolution in orioles (*Icterus*): repeated convergence and reversal in patterns. *Evolution* 2000, 54(6):2119-2133.

O`Neill KM: Solitary wasps: behavior and natural history. 1st ed. Ithaca: Cornell University Press, 2001.

Ord TJ, Martins EP: Tracing the origins of signal diversity in anole lizards: phylogenetic approaches to inferring the evolution of complex behaviour. *Animal Behaviour* 2006, 71(6):1411-1429.

Pagel M: Inferring the historical patterns of biological evolution. *Nature* 1999, 401(6756):877-884.

Palmer CA, Watts RA, Gregg RG, McCall MA, Houck LD, Highton R, Arnold SJ: Lineage-specific differences in evolutionary mode in a salamander courtship pheromone. *Molecular Biology and Evolution* 2005, 22(11):2243-2256.

Panhuis TM, Butlin R, Zuk M, Tregenza T: Sexual selection and speciation. *Trends in Ecology & Evolution* 2001, 16(7):364-371.

Paradis E, Claude J, Strimmer K: APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 2004, 20:289-290.

Penagos-Arévalo AC, Billen J, Sarmiento CE: Uncovering head gland diversity in neotropical Polistinae wasps (Hymenoptera, Vespidae): Comparative analysis and description of new glands. *Arthropod Structure & Development* 2015, 44(5):415-425.

Pietrobon TAO, Caetano FH: Ultramorphology and histology of the ectal mandibular gland in *Polistes versicolor* (Olivier) (Hymenoptera: Vespidae). *Cytologia* 2003, 68(1):89-94.

Polidori C, Boesi R, Isola F, Andrietti F: Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): The role of prey size. *European Journal of Entomology* 2005, 102(4):801-804.

Polidori C, Federici M, Papadia C, Andrietti F: Nest sharing and provisioning activity of females of the digger wasp, *Cerceiis rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 2006, 73(1):55-65.

Pomiankowski A, Iwasa Y: Runaway ornament diversity caused by Fisherian sexual selection. *Proceedings of the National Academy of Sciences of the United States of America* 1998, 95(9):5106-5111.

Porter ML, Crandall KA: Lost along the way: the significance of evolution in reverse. *Trends in Ecology and Evolution* 2003, 18(10):541-547.

Potti J, Canal D: Heritability and genetic correlation between the sexes in a songbird sexual ornament. *Heredity* 2011, 106:945-954.

Price JJ, Lanyon SM: Patterns of song evolution and sexual selection in the oropendolas and caciques. *Behavioral Ecology* 2004, 15(3):485-497.

Price JJ, Friedman NR, Omland KE: Song and plumage evolution in the new world orioles (*Icterus*) show similar lability and convergence in patterns. *Evolution* 2007, 61(4):850-863.

Prum RO: Phylogenetic tests of alternative intersexual selection mechanisms: Trait macroevolution in a polygynous clade (Aves: Pipridae). *The American Naturalist* 1997, 149(4):668-692.

Pulawski WJ: Number of species. In: Catalog of Sphecidae. California Academy of Sciences, San Francisco. 2016a. http://researcharchive.calacademy.org/research/entomology/entomology_resources/hymenoptera/Sphecidae/number_of_species.pdf. Accessed 30 Oct 2016.

Pulawski WJ: *Philanthus* - List of species. In: Catalog of Sphecidae. California Academy of Sciences, San Francisco. 2016b. http://researcharchive.calacademy.org/research/entomology/entomology_resources/hymenoptera/sphecidae/genera/Philanthus.pdf. Accessed 30 Oct 2016.

Puniamoorthy N, Su KFY, Meier R: Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae: Diptera). *BMC Evolutionary Biology* 2008, 8.

R Core Team: R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna. 2017. http://www.R-project.org.

Raffa KF, Hobson KR, LaFontaine S, Aukema BH: Can chemical communication be cryptic? Adaptations by herbivores to natural enemies exploiting prey semiochemistry. *Oecologia* 2007, 153(4):1009-1019.

Ritchie MG: Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics* 2007, 38:79-102.

Ruther J, Matschke M, Garbe LA, Steiner S: Quantity matters: male sex pheromone signals mate quality in the parasitic wasp *Nasonia vitripennis*. *Proceedings of the Royal Society B-Biological Sciences* 2009, 276(1671):3303-3310.

Safran RJ, Scordato ESC, Symes LB, Rodriguez RL, Mendelson TC: Contributions of natural and sexual selection to the evolution of premating reproductive isolation: A research agenda. *Trends in Ecology & Evolution* 2013, 28(11):643-650.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A: Fiji: An open-source platform for biological-image analysis. *Nature Methods* 2012, 9(7):676-682.

Schmid B, Schindelin J, Cardona A, Longair M, Heisenberg M: A high-level 3D visualization API for Java and ImageJ. *BMC Bioinformatics* 2010, 11.

Schmidt JO, Oneill KM, Fales HM, McDaniel CA, Howard RW: Volatiles from mandibular glands of male beewolves (Hymenoptera, Sphecidae, *Philanthus*) and their possible roles. *Journal of Chemical Ecology* 1985, 11(7):895-901.

Schmitt T, Strohm E, Herzner G, Bicchi C, Krammer G, Heckel F, Schreier P: (S)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *Journal of Chemical Ecology* 2003, 29(11):2469-2479.

Schmitt T, Herzner G, Weckerle B, Schreier P, Strohm E: Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. *Apidologie* 2007, 38(2):164-170.

Scordato ESC, Symes LB, Mendelson TC, Safran RJ: The role of ecology in speciation by sexual selection: A systematic empirical review. *Journal of Heredity* 2014, 105:782-794.

Seddon N, Botero CA, Tobias JA, Dunn PO, MacGregor HEA, Rubenstein DR, Uy JAC, Weir JT, Whittingham LA, Safran RJ: Sexual selection accelerates signal evolution during speciation in birds. *Proceedings of the Royal Society B-Biological Sciences* 2013, 280(1766).

Seehausen O, van Alphen JM: Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecology Letters* 1999, 2(4):262-271.

Slessor KN, Kaminski LA, King GGS, Borden JH, Winston ML: Semiochemical basis of the retinue response to queen honey bees. *Nature* 1988, 332(6162):354-356.

Smadja C, Butlin RK: On the scent of speciation: The chemosensory system and its role in premating isolation. *Heredity* 2009, 102(1):77-97.

Soroker V, Vienne C, Hefetz A, Nowbahari E: The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 1994, 81:510-513.

Soroker V, Hefetz A, Cojocaru M, Billen J, Franke S, Francke W: Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 1995, 20:323-329.

Steiger S, Schmitt T, Schaefer HM: The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society B-Biological Sciences* 2010, 278(1708):970-979.

Steiner AL: Observations on spacing, aggressive and lekking behavior of digger wasp males of *Eucerceris flavocincta* (Hymenoptera: Sphecidae, Cercerini). *Journal of the Kansas Entomological Society* 1997, 51(3):492-498.

Stökl J, Herzner G: Morphology and ultrastructure of the allomone and sex-pheromone producing mandibular gland of the parasitoid wasp *Leptopilina heterotoma* (Hymenoptera: Figitidae). *Arthropod Structure & Development* 2016, 45(4):333-340.

Strohm E: Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). Berlin: Verlag Dr, Köster, 1995.

Strohm E, Lechner K: Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 2000, 59(1):183-191.

Strohm E, Linsenmair KE: Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 1999, 47(1-2):76-88.

Strohm E, Linsenmair KE: Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 2001, 26(2):198-203.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development*. 2007, 36(2):113-122.

Strohm E, Kaltenpoth M, Herzner G: Is the postpharyngeal gland of a solitary digger wasp homologous to ants? Evidence from chemistry and physiology. *Insectes Sociaux* 2010, 57(3):285-291.

Symonds MR, Elgar MA: The evolution of pheromone diversity. *Trends in Ecology & Evolution* 2008, 23(4):220-228.

Symonds MR, Moussalli A, Elgar MA: The evolution of sex pheromones in an ecologically diverse genus of flies. *Biological Journal of the Linnean Society* 2009, 97(3):594-603.

Tesfaye T, Getachew B, Worede M: Morphological diversity in tetraploid wheat landrace populations from the central highlands of Ethiopia. *Hereditas* 1991, 114(2):171-176.

Tobias JA, Montgomerie R, Lyon BE: The evolution of female ornaments and weaponry: Social selection, sexual selection and ecological competition. *Philosophical Transactions of the Royal Society B* 2012, 367: 2274-2293.

van Doorn GS, Dieckmann U, Weissing FJ: Sympatric speciation by sexual selection: a critical reevaluation. *The American Naturalist* 2004, 163(5):709-725.

van Doorn GS, Edelaar P, Weissing FJ: On the origin of species by natural and sexual selection. *Science* 2009, 326(5960):1704-1707.

Vinson SB, Williams HJ, Frankie GW, Wheeler JW, Blum MS, Coville RE: Mandibular glands of male *Centris adani* (Hymenoptera, Anthophoridae) - Their morphology, chemical constituents, and function in scent marking and territorial behavior. *Journal of Chemical Ecology* 1982, 8(2):319-327.

Vinson SB, Williams HJ, Frankie GW, Coville RE: Comparative morphology and chemical contents of male mandibular glands of several *Centris* species (Hymenoptera, Anthophoridae) in Costa Rica. *Comparative Biochemistry and Physiology A* 1984, 77(4):685-688.

Wagner CE, Harmon LJ, Seehausen O: Ecological opportunity and sexual selection together predict adaptive radiation. *Nature* 2012, 487(7407):366-370.

Weber MG, Mitko L, Eltz T, Ramírez SR: Macroevolution of perfume signalling in orchid bees. *Ecology Letters* 2016, 19(11):1314-1323.

Weiss K, Strohm E, Kaltenpoth M, Herzner G: Comparative morphology of the postpharyngeal gland in the Philanthinae (Hymenoptera, Crabronidae) and the evolution of an antimicrobial brood protection mechanism. *BMC Evolutionary Biology* 2015, 15.

Weissing FJ, Edelaar P, van Doorn GS: Adaptive speciation theory: a conceptual review. *Behavioral Ecology and Sociobiology* 2011, 65(3):461-480.

West-Eberhard MJ: Sexual selection, social competition, and speciation. *Quarterly Review of Biology* 1983, 58(2):155-183.

Wiens JJ: Widespread loss of sexually selected traits: how the peacock lost its spots. *Trends in Ecology & Evolution* 2001, 16(9):517-523.

Williams HJ, Vinson SB, Frankie GW, Coville RE, Ivie GW: Morphology, chemical contents and possible function of the tibial gland of males of the Costa Rican solitary bees *Centris nitida* and *Centris trigondoides subtarsata* (Hymenoptera, Anthophoridae). *Journal of the Kansas Entomological Society* 1984, 57(1):50-54.

Winston ML, Slessor KN: The essence of royalty - Honey-bee queen pheromone. *American Scientist* 1992, 80(4):374-385.

Zimmermann D, Vilhelmsen L: The sister group of Aculeata (Hymenoptera) - evidence from internal head anatomy, with emphasis on the tentorium. *Arthropod Systematics & Phylogeny* 2016, 74(2):195-218.

# CHAPTER 6

# GENERAL DISCUSSION

> *"Yes, I agree that we have limited understanding how morphological diversity came about and what explains its intricate patterns and conservation, but lack of understanding does not prove that there is nothing to be understood. […] So let us assume that we can agree on the latter point and proceed from there."*
>
> *Günter Wagner, Biology & Philosophy 30(6) (2015)*

The present PhD thesis aimed at deciphering the evolution of the head glands of male and female Philanthinae. The first chapters (2-4) present comprehensive evidence that in females of the tribe Philanthini, but probably not in the other tribes of the subfamily, the postpharyngeal gland (PPG) is under stabilizing selection due to its involvement in a unique antimicrobial parental care behavior. Chapter 5 deals with the evolution of interspecific diversity in the males' pheromone glands under sexual selection and presents statistical evidence for the diversifying effect of sexual selection on trait evolution. In this chapter, I will bring together considerations on females' PPGs and the evolution of prey embalming, the evolution of male head glands in the context of pheromone-based mate choice and preliminary results on interspecific diversity in pheromone chemistry, as well as the possible concerted evolution of male and female head glands in the Philanthinae.

## 6.1 The postpharyngeal gland and antimicrobial brood defense

### 6.1.1 The postpharyngeal glands of Hymenoptera

The PPG is a quite unusual gland in insects and, as far as is known, does only occur in Hymenoptera. In the Philanthinae, the PPG has first been detected in both sexes of the European beewolf, *Philanthus triangulum* by Herzner et al. (2007a) and Strohm et al. (2007) and subsequently in females of the closely related *Trachypus boharti* and *Trachypus elongatus* (Herzner et al. 2013), but is now known to be present in all studied taxa of the subfamily (chapters 2, 4, and 5). Before its discovery in beewolves, the PPG had only been described for ants and was considered to be idiosyncratic to the Formicidae (e.g. Billen 1990, Hölldobler & Wilson 1990,

Jackson & Morgan 1993, Lenoir et al. 1999; see also Martin & Drijfhout 2009) where its main function is the formation, distribution, and maintenance of the chemical identity of a colony (the so-called 'Gestalt' odor; Crozier & Dix 1979) that is crucial for nestmate recognition (Hefetz et al. 1992, 1996, Soroker et al. 1994, 1995a, Vienne et al. 1995, Dahbi et al. 1998, Lenoir et al. 2001, Akino et al. 2004).

Besides ants and Philanthinae, a PPG has been found in the basal aculeate wasp *Ampulex compress* (Hymenoptera, Ampulicidae) (Herzner et al. 2011a), and there is evidence that also female cuckoo wasps (Hymenoptera, Chrysididae), a very basal taxon not closely related to the Apoidea (Melo 1999, Ohl & Bleidorn 2006, Debevec et al. 2012), possess a PPG (G. Herzner, E. Strohm, W. Goettler, *personal communication*). On the other hand, bees, which are closely related to the Crabronidae (e.g. Melo 1999, Ohl & Bleidorn 2006, Debevec et al. 2012), apparently lack a PPG. Hence, the occurrence of a PPG is undoubtedly more widespread among Hymenoptera than previously thought, but its phylogenetic distribution and origin remains uncertain.

Among different taxa of the Hymenoptera, and even among female Philanthinae (chapters 2 and 4), there are considerable differences in PPG morphology. In females of the tribe Philanthini, as well as in many ants (Peregrine et al. 1973, Soroker et al. 1995b, Niculita et al. 2007), the PPG is a large, complex reservoir that has a very conspicuous 'glove-like' form, with many 'fingers' branching off a common root, and occupies most of the front part of the head capsule anterior to the brain (Strohm et al. 2007, Herzner et al. 2013; chapter 2). The glands of *A. compressa*, the most closely related taxon for which a PPG has yet been described, however, constitutes only simple tubular evaginations of the pharynx, located behind the brain (Herzner et al. 2011a). Interestingly, some phylogenetic studies have recently suggested that ants might be more closely related to crabronid wasps than previously thought, actually forming the sister group of the Apoidea (Johnson et al. 2013, Danforth 2013, Branstetter et al. 2017), which would explain the largely similar PPG morphology in female Philanthini and many ants (Strohm et al. 2010).

### 6.1.2 Prey embalming in the Philanthini

Though all female Philanthinae investigated thus far possess PPGs, there are marked morphological differences among different phylogenetic clades (chapters 2 and 4). While females of the tribe Philanthini, the so-called beewolves, possess the large complex reservoirs described above, the females' PPGs in the other two tribes of the subfamily, the Cercerini and

Aphilanthopsini, consist of only simple tube-shaped evaginations of the pharynx that rather resemble the PPG of the basal *A. compressa* (Herzner et al. 2011a). Accordingly, female Philanthini were clearly separated from the other two tribes by a categorical principal components analysis based on female PPG morphology (Figure 6.1).

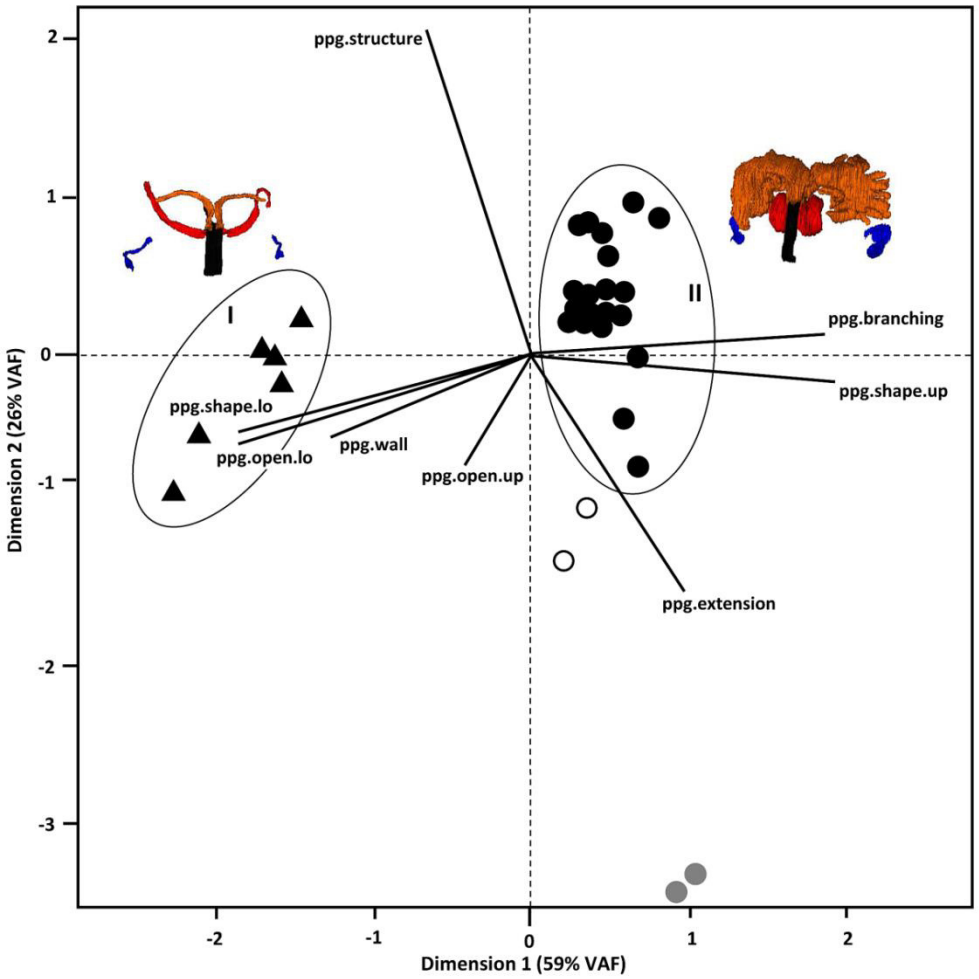


**Figure 6.1** Categorical principal components analysis (CATPCA) of postpharyngeal gland (PPG) morphology of female Philanthini (circles) as well as Aphilanthopsini and Cercerini (triangles) (morphological data from chapters 2 and 4). The first two dimensions (VAF, variance accounted for by the respective dimension), clearly separate the Aphilanthopsini/Cercerini from all species of the Philanthini (ellipses; exemplary 3D-reconstructions: (I) *Cerceris quinquefasciata*, (II) *Philanthus barbiger*). Only two *Trachypus* species (grey circles) deviate from the other Philanthini in that they do not possess the lower part of the PPG reservoir (see chapter 2), present in all other species (represented by character 'Structure of the PPG'). When repeating the CATPCA without character 'Structure of the PPG' these two species also cluster close to the other Philanthini (open circles). Vectors: component loadings of eight included morphological characters. Abbreviations of the morphological characters (Values in parentheses are Eigenvalues of the characters): ppg.branching, Number of lobes of the PPG (0.999); ppg.extension, Lateral extension of the PPG relative to the head capsule (0.872); ppg.open.lo, Numbers of openings of the lower part of the PPG to the pharynx (0.985); ppg.open.up, Numbers of openings of the upper part of the PPG to the pharynx (0.187); ppg.shape.lo, shape of the lower part of the PPG (1.013); ppg.shape.up, shape of the upper part of the PPG (1.023); ppg.structure, Overall structure of the PPG (1.257); ppg.wall, Structure of the inner walls of the PPG (0.496).

In female Philanthini, not only PPG morphology, but also the chemical composition of their secretions show very little interspecific variation, with unsaturated long-chain hydrocarbons (HCs) generally being the predominant compound class (chapter 3). In *P. triangulum*, and likely in all other Philanthini, these unsaturated HCs are crucial for a unique antimicrobial brood care mechanism, the embalming of their paralyzed prey with PPG secretion (Herzner & Strohm 2007, Herzner et al. 2007b, 2013; chapter 2), which reduces water condensation on the prey's surface und, thus, restrains the growth of mold fungi (Herzner & Strohm 2007, Herzner et al. 2007b, 2011b). The uniform PPG morphology and chemistry of female Philanthini has, thus, most likely evolved under stabilizing selection constraining the size and complexity of the gland reservoirs, and especially of the chemical composition of their secretions.

*Evolution of chain length*

Despite the highly uniform composition of substance classes in PPG secretion among female Philanthini, however, there were some differences regarding average chain length (ACL, calculated as $N = \sum a_i p_i$ with $a_i$ being a component with a backbone length of i carbon atoms and $p_i$ being the summed proportion of all substances with i carbon atoms) of PPG compounds between phylogenetic lineages (mean ACL Afrotropical/Palearctic *Philanthus* (N=5): 25.5, *Trachypus* (N=3): 27.0, North American *Philanthus* (N=16): 26.7), although these differences were not statistically significant (Weiss et al., *unpublished data*). A similar situation is found in the formicine ant genus *Lasius*. Throughout this genus, 3-methylalkanes are thought to act as conserved queen pheromones, but chain lengths of 3-methylalkanes vary considerably across species (Holman et al. 2013). As chain length of a given HC compound seems to be independent from the insertion of functional groups during biosynthesis (Blomquist & Bagnères 2010), the authors suggest that 3-methylalkanes are conserved as queen pheromones by constrains in worker odorant perception, while chain length might be under directional selection, for instance by different environmental temperatures and humidity (Holman et al. 2013). Accordingly, in female Philanthini, the general prevalence of unsaturated HCs is likely constrained by their function in prey preservation, while chain length differences might either be selected by diverging abiotic conditions among geographically isolated lineages, or alternatively represent non-adaptive phylogenetic changes.

*Costs of prey embalming*

To counter pathogenic microbes, insects possess a potent and complex immune system including innate responses and also a kind of acquired immunity (reviewed in Schmid-Hempel 2005, Siva-Jothy et al. 2005). This 'internal' immune system is more and more broadened to what has

recently been termed 'external immune defense' (Otti et al. 2014). In insects, external immune defense may include behavioral adaptations (e.g. Elliot et al. 2002, Cremer et al. 2007, Ormond et al. 2011), but in particular exocrine antimicrobial secretions employed to disinfect the insect's own body surface and its environment including brood and nestmates, food, and nest substrate (e.g. Otti et al. 2014, Tragust 2016). In this sense, prey embalming can also be seen as part of the female's extended immune defense.

Any form of antimicrobial defense, however, goes with some costs. Following Kraaijeveld et al. (2002) and Sandland and Minchella (2003), there can be two different levels of costs. On the one hand, physiological (or inducible) costs arise from withdrawing limited resources from other physiological processes upon actual defense (i.e. after actual infection) (e.g. Siva-Jothy et al. 2001); on the other hand, constitutive (or evolutionary) costs are associated with negative genetic correlations between a "standing immune defense" and other fitness components, even in the absence of an acute threat by microbes or parasites (Kraaijeveld et al. 2002).

Considering prey embalming in the Philanthini, the maintenance of a large and complex PPG as a storage organ for embalming secretion, as well as the metabolic basis for effective uptake of HCs may be considered as evolutionary costs, whereas physiological trade-offs may chiefly include the continuous production of high amounts of HCs (Herzner et al. 2011b). Between the three species known to embalm their prey, *P. triangulum*, *Philanthus gibbosus*, and *T. elongatus*, the total amount of PPG secretion applied to a prey item varies considerably (Herzner & Strohm 2007, Herzner et al. 2007b, 2013; chapter 2), suggesting interspecific differences in the amount of HCs needed for effective embalming. Possible selective factors accounting for such differences may include abiotic factors like humidity, temperature, or nest depth, but also differences in prey spectrum (as discussed in chapter 2). Though the actual nature of the selective forces that favor the evolution of prey embalming remain to be determined, we may yet assume that this unique antimicrobial parental care strategy is shared by all members of the Philanthini.

### 6.1.3 Evolutionary origin of prey embalming

Regarding the occurrence of prey embalming, the picture is less clear for the other tribes of the Philanthinae. Though scarce, available data at present suggest that especially the genus *Cerceris* might provide a key to the evolution of prey embalming. *Cerceris* is not only the largest genus of the Philanthinae, comprising well over 850 species world-wide (Pulawski 2017), it is also the only philanthine genus in which different species use taxa from different insect orders as prey. While

most *Cerceris* use Coleoptera (chiefly weevils and buprestids) as larval provision, some species hunt for bees (e.g. Bohart & Menke 1976, Polidori et al. 2005, 2006). Among the three philanthine tribes there are considerable differences in prey spectrum, which might influence the need for antimicrobial protection due to possible differences in the susceptibility of different prey taxa to microbial infestation (as discussed in chapter 2). Therefore, one might expect to find differences in antimicrobial strategies among bee- and beetle-hunting *Cerceris*. Yet, the uniform PPG morphology among 'prey types', with both bee- and beetle-hunters possessing small and simple glands (chapter 4), does not corroborate this hypothesis.

The analysis of PPG chemistry in *Cerceris*, however, yielded equivocal results. The chemical profile of the beetle-hunting *Cerceris arenaria* was markedly dominated by methyl-branched HCs, but possessed only small proportions of unsaturated compounds (chapter 3). By contrast, in two bee-hunting species, *Cerceris hortivaga* and *Cerceris rybyensis*, unsaturated HCs are by far the predominant substance class, accounting for 79 ± 0.5% (mean ± standard deviation) of the total substance amount in *C. hortivaga* (N=2) and 60 ± 14% in *C. rybyensis* (N=7) (Weiss et al., *unpublished data*). Thus, though not differing in gland morphology, the PPG chemistry of bee-hunting *Cerceris* seems to be markedly different from their beetle-hunting congeners, but rather resembles that of the Philanthini. Moreover, preliminary results by Wurdack et al. (*personal communication*) suggest, that provisioned bees taken from the nests of bee-hunting *Cerceris* carry higher amounts substances on their cuticle that are characteristic for the surface chemistry of the respective *Cerceris* species but not for control bees without contact to the wasp. The surface chemistry of provisioned beetles taken from nests of beetle-hunting *Cerceris*, on the other hand, is not changed as compared to controls (Wurdack et al., *personal communication*). This might point to differences in prey treatment among bee- and beetle-hunting *Cerceris*, suggesting that prey-type actually is an important factor in shaping PPG chemistry.

Taken together, though likely common to all Philanthini, at present our data do not allow for inferences about the evolutionary origin and phylogenetic distribution of prey embalming in the Philanthinae. More data on PPG morphology and chemistry, as well as chemical investigations of provisioned prey will be needed for members of the other philanthine tribes, as well as other, more basal taxa of the Apoidea, and even other Hymenopteran lineages, to clarify the evolutionary history of this extraordinary antimicrobial brood protection mechanism.

## 6.2 Male head glands and pheromone communication

As compared to females, the head gland morphology of male Philanthinae shows significantly more interspecific variation (chapter 5). As described for *P. triangulum*, both the PPG and the mandibular gland (MG) of males function as production and/or storage organs for a marking pheromone (Kroiss et al. 2006, Herzner et al. 2007a, Goettler & Strohm 2008) that is used to establish small territories and attract receptive females (Alcock 1975, Evans & O'Neill 1985, 1988, Clarke et al. 2001, Strohm & Lechner 2000, O'Neill 2001, Kroiss et al. 2010). Therefore, the immense morphological variation in male pheromone glands can most likely be attributed to sexual selection, which is often assumed to promote the rapid divergence of traits, even among closely related species (e.g. Seehausen & van Alphen 1999, Kopp & True 2002, Hosken & Stockley 2004, Emlen et al. 2005, Ord & Martins 2006, Arnegard et al. 2010, Wagner et al. 2012, Seddon et al. 2013, Bacquet et al. 2015). A detailed discussion of the morphological diversity of male head glands, its intricate phylogenetic patterns, and the possible evolutionary history of gland characters is provided in chapter 5. Yet, what actually causes this morphological diversity remains elusive.

In fact, selection by female choice does not directly target the morphology of male pheromone glands, but rather their secretions. Since, in the Philanthinae, after a female has alighted in a male's territory, mating seems to take place without any further male courtship (e.g. Evans & O'Neill 1988, Kroiss et al. 2010), the male pheromone likely is the only means of female mate choice and should, thus, be under strong selection to maximize its attractiveness. Consequently, to understand the origin of the morphological diversity in male head glands, one has to consider selection acting on male pheromones. In the following sections I will therefore focus on what selection factors might shape male pheromones, present some preliminary results on pheromone chemical diversity, and discuss how pheromone composition might impact gland morphology.

### 6.2.1 Possible functions of male pheromones in the Philanthinae

Regarding the evolution of male pheromones, there may be two principle levels of selection, species recognition and mate assessment (e.g. Blows & Allan 1998, Lewis et al. 2004; see also Anderson 1994). In the European beewolf, *P. triangulum*, as in other insects (Thornhill 1992, Moore 1994, Iyengar & Eisner 1999, Iyengar et al. 2001, Jones et al. 2000, Beeler et al. 2002, Hine et al. 2002, Rantala et al. 2002, 2003, Blaul & Ruther 2011, Chemnitz et al. 2015), the male pheromone offers the potential for adaptive female choice as it may contain a wealth of

information on male quality, including age (Kaltenpoth & Strohm 2006), size (Strohm et al., *unpublished data*), geographic origin and kinship (Herzner et al. 2006, Kaltenpoth et al. 2007), as well as developmental conditions (Roeser-Mueller et al. 2010). In the Philanthinae, the geographic origin of a male and the degree of relatedness may be of special importance for adaptive mate choice. Hymenoptera in general have an arrhenotokous haplodiploid sex determination mechanism and in many lineages, including Apoidea, this mechanism is based on a single locus (single-locus complementary sex-determination, sl-CSD) (e.g. Cook 1993, Beye et al. 2003, van Wilgenburg et al. 2006, Heimpel & de Boer 2008). In arrhenotokous specie, unfertilized (haploid) eggs develop into males, while fertilized (diploid) eggs normally develop into females. Yet, diploid individuals that are homozygous at the sex determination locus develop into diploid males, which are generally sterile (Cook 1993, Cook & Crozier 1995, Heimpel & de Boer 2008). Inbreeding increases the degree of homozygosity in a population and, thus, the chance of diploid sons, especially in species with sl-CSD. Moreover, since female Philanthinae most likely are monandrous (Evans & O'Neill 1988), they would not be able to offset the high costs of mating with a closely related male. Assessment of relatedness to a potential mate may consequently be a relevant factor in the evolution of the male pheromone in the Philanthinae.

Yet, mating with a male from the wrong species would lead to even greater fitness losses. Therefore, another crucial aspect of sexual signals is species recognition (Andersson 1994, Coyne & Orr 2004), as has, for instance, been demonstrated for insect pheromone communication (Danci et al. 2006, Groot et al. 2006, Dyer et al. 2014, Juárez et al. 2016). Owing to special characteristics of their mating system, the species specificity of pheromone signals may be especially important in the Philanthinae. Following Kroiss et al. (2010), the mating system of the Philanthinae can be described as 'hotspot' lek polygyny (Höglund & Alatalo 1995, Shelly & Whittier 1997), meaning that male territories aggregate where females are likely to occur, for instance due to foraging or nesting (a hotspot). At least in the Philanthini, males of different species often co-occur in what might be called 'multi-species leks' (Evans & O'Neill 1988; G. Herzner, E. Strohm, M. Kaltenpoth, *unpublished data*). The same may hold true of specie from other tribes (Alcock 1975).

This may lead to what is known as the 'cocktail party problem' in acoustic communication (Cherry 1953). The cocktail party problem refers to the problem that a listener, to be able to follow a conversation in a noisy environment (be it a cocktail party or a multi-species animal chorus), has first to selectively recognize and then attend to sounds originating from one source among all other competing sound sources (Haykin & Chen 2005, Bee & Micheyl 2008, McDermott 2009). A

similar problem, a 'chemical cocktail party problem', may occur to female Philanthinae facing simultaneously signaling males at a multi-species lek. To locate conspecific males, a female first has to detect the different components forming its species' pheromone and then recognize the source of this specific blend among other sources, i.e. heterospecific males possibly emitting similar component mixtures.

### 6.2.2 Selection for interspecies diversity in male pheromones?

The frequent spatial and temporal co-occurrence of signaling males of different species may have different consequences for the evolution of male pheromones in the Philanthinae. For instance, it might select for pheromone components that are especially easily detected by conspecific females. Moreover, it should favor the evolution of chemical signals that are easily discriminable against the environmental background (Schaefer & Ruxton 2015), with the pheromone signals of other sympatric species belonging to this background.

The data that have hitherto been published on pheromone chemistry in the Philanthinae support these predictions. In *P. triangulum*, the male pheromone is indeed dominated by one component for which females are very sensitive due to a preexisting sensory bias (see section 6.2.3 below), the unsaturated alcohol (*Z*)-11-eicosen-1-ol (Schmitt et al. 2003, Herzner et al. 2005, Kroiss et al. 2006), and in five North American species that have been investigated earlier (Schmidt et al. 1985, McDaniel et al. 1987, 1992), *Philanthus barbatus*, *Philanthus basilaris*, *Philanthus bicinctus*, *Philanthus crabroniformis*, and *Philanthus pulcher*, the male pheromone is likewise always dominated by a few main components that show considerable qualitative difference among species (Schmidt et al. 1985, McDaniel et al. 1987, 1992).

The analysis of our own gas chromatography/mass spectrometry (GC/MS) data for the previously investigated species confirmed these earlier results (Weiss et al., *unpublished data*). Moreover, preliminary comparative GC/MS analyses of the pheromone profiles of another 32 species (obtained by extraction of whole heads), including North American, Afrotropical, Palearctic, and Asian *Philanthus* species, as well as males of the Neotropical subgenus *Trachypus*, revealed the same general patter in all species. All pheromone profiles were largely dominated by one to a few main compounds with the main peak alone in most cases representing on average between 35 and 80 % of the total substance amount (Weiss et al., *unpublished data*). To date, we have not completely identified any of these components. Yet, the comparison of mass spectra and tentative substance identities obtained from a commercial MS library (NIST, Gaithersburg, MD,

USA) of the main components (i.e. those substances, or rather GC peaks, that represented >25% of the total substances of at least in one analyzed individual of a species; between one to eight peaks per species), suggested that, though the same substances were detected in different species, not one species pair shares the same combination of main peaks (Weiss et al., *unpublished data*). In all but one investigated species, pheromones were dominated by carboxylic acids, esters, alcohols and aldehydes, and/or methyl-branched HCs, while linear HCs occurred in relatively low proportions. Interestingly, however, head extracts of the North American *Philanthus albopilosus*, the only philanthine species thus fare known that is not territorial (Evans & O'Neill 1988) and consequently does not possess elaborate head glands (chapter 5), were largely dominated by unsaturated (62 %) and saturated (31 %) linear HCs (Weiss et al., *unpublished data*), thus closely resembling the chemical profiles of female *Philanthus* (chapter 3).

Though we currently do not know whether the detected main compounds actually have a function in pheromone communication, the apparent general pattern of pheromone chemistry seems highly suitable to mediate species isolation even among closely related, sympatric species. Interestingly, males of three species of the genus *Eucerceris*, belonging to the philanthine tribe Cercerini, all possess the same main pheromone compound, (*Z*)-3-hexenyl-(*R*)-3-hydroxybutyrate (Clarke et al. 2001), suggesting that in these species species-specificity is either achieved by minor pheromone components, or that they have evolved a different form of species recognition mechanism, Alternatively, there might be no need for species recognition in this genus, for example due to the lack of sympatric occurrence (but see Alcock 1975).

### 6.2.3 Possible factors shaping pheromone diversity in the Philanthinae

The need for effective species recognition in communally lekking Philanthinae may likely be the ultimate evolutionary cause of the observed large interspecific differences in male pheromones. Yet, what are the different selection factors that actually shape these differences?

*Female sensory biases*

One factor known to influence the evolution of mating signals is the exploitation of preexisting receiver biases (e.g. Endler 1992, Ryan 1998, Ryan & Cummings 2013). This mechanism is thought to explain the high susceptibility of females to sexual signals and has, for instance, been invoked for the evolution of visual signals (Boughman 2001, 2002, Seehausen et al. 2008), but also of pheromone communication (Buchinger et al. 2013). In the European beewolf, *P. triangulum*, the main constituent of the male pheromone, (*Z*)-11-eicosen-1-ol, forms also part of the alarm

pheromone of honeybees, *Apis mellifera*, the sole prey of females. Females use trace amounts of (*Z*)-11-eicosen-1-ol present on the cuticle of forging worker bees to identify their prey and, thus, probably are highly sensitive to this substance (Herzner et al. 2005, Schmitt et al. 2007, Steiger et al. 2011). Also in other species, especially in those in which females are prey specialists, like the Indian *Philanthus basalis* that is specialized on the Indian honeybee (*Apis cerana indica*) (Krombein 1981), or the South American *T. boharti* that is specialized on male *Scaptotrigona postica* bees (Koedam et al. 2009, 2011), the males' pheromone may include specific substances that are also used by females to locate and/or identify their prey ('sensory trap', e.g. Christy 1995). In *T. boharti*, for instance, females likely rely on quantitative differences in bees' cuticular HCs to distinguish male *S. postica* from workers (Koedam et al. 2011).

Yet, most beewolves have a broader prey spectrum, often including bees from different families, and sometimes even other aculeate wasps (Evans & O'Neill 1988). Nevertheless, in these species males might exploit more general sensory biases, like for instance a high sensitivity for certain substance classes that are common in a groups of often used prey species, rather than specific single substances ('sensory exploitation', e.g. Ryan 1990).

*Heterospecific interference*

Interspecific differences in chemical signals may either evolve by the gradual accumulation of small changes, leading to closely related species having similar pheromone blends, or by saltatorial shifts in pheromone composition, resulting in large differences between close relatives (Symonds & Elgar 2008, Smadja & Butlin 2009). In signals mediating species recognition and consequently prezygotic reproductive isolation, however, there should be strong stabilizing selection against even small changes, since individuals deviating from the species 'norm' would suffer reduced fitness (Symonds & Elgar 2008, Smadja & Butlin 2009). Yet, although this should all the more hold true for saltatorial shifts, many examples can be quoted that show large differences in pheromone composition among closely related species, suggesting the rapid evolution of pheromone chemistry, including the species specific aggregation pheromones of bark beetles (Symonds & Elgar 2004), the male pheromone of *Bactrocera* fruit flies (Symonds et al. 2009), and cuticular HCs of *Drosophila* that are involved in sexual communication (reviewed in Ferveur 2005).

The rapid evolution of pheromone diversity as such may not be surprising, given that in insects the genetic control of pheromone production is often quite simple (e.g. Löfstedt 1990, Marcillac et al. 2005, Tabata & Ishikawa 2005, Domingue et al. 2006, Albre et al. 2012, Niehuis et al. 2013)

and already small genetic changes in biosynthetic pathways can result in large differences in pheromone composition, even among closely related species (Roelofs et al. 2002, Niehuis et al. 2013, Buček et al. 2015). Since the same likely applies to the evolution of pheromone receptors (Hansson et al. 1987, Roelofs et al. 1987, Marcillac et al. 2005, Gould et al. 2010, Leary et al. 2012), there might be mutant receivers in a population that are able to respond to a saltatorial change in the pheromone of a mutant sender. For example, Roelofs et al. (2002) demonstrated that in European corn borer moths, *Ostrinia nubilalis*, rare males exist that respond to both the pheromone blend of conspecific females, as well as a newly evolved pheromone component of the Asian corn borer, *Ostrinia furnacalis*. Thus, the saltatorial evolution of species specific pheromone signals is indeed feasible, possibly leading to instantaneous reproductive isolation among populations.

Especially when closely related species co-occur at the same mating sites, as is often the case in beewolves (Evans & O'Neill 1988; G. Herzner, E. Strohm, M. Kaltenpoth, *unpublished data*), selection against hybridization may favor the rapid evolution of major shifts in pheromone composition due to character displacement and reinforcement among sympatric species (Danci et al. 2006 Groot et al. 2006, Dyer et al. 2014, Bacquet et al. 2015, Juárez et al. 2016, Weber et al. 2016). Heterospecific interference may thus contribute to the high interspecific diversity in pheromone composition in the Philanthinae.

*Pheromone exploitation*

Besides their intended function in intraspecific communication, pheromones can also be exploited as kairomones by predators or parasitoids (e.g. Stowe et al. 1995, Zuk & Kolluru 1998, Haynes & Yeargan 1999), which might lead to adaptations in chemical signals (Aldrich et al. 1989, Raffa & Dahlsten 1995). There is no evidence, however, that predators or parasites attacking adult beewolves use chemical cues for prey/host location. Yet, there are numerous brood parasitoids associated with beewolves that can cause severe losses in brood (e.g. Strohm et al. 2001). Due to the generally close proximity of male territories and active female nesting sites (e.g. Evans & O'Neill 1988), male pheromones might also serve as cues for brood parasitoids. The parasitic wasp *Hedychrum rutilans*, parasitizing nests of *P. triangulum*, most likely uses host derived HCs in excavated nest material for close-range nest detection (Kroiss et al. 2008). Though in *H. rutilans* long-distance attraction is likely due to visual cues, it may be theoretically feasible that olfactory-oriented brood parasitoids, exploit certain pheromone components of nearby conspecific males in host search, as has been demonstrated for other egg and brood parasitoids (e.g. Colazza et al. 1997, Hilker et al. 2000, Arakaki et al. 2011; see also Fatouros et al. 2008). The presence of

pheromone-exploiting brood parasitoids in some populations might impair the evolution of different pheromone components and might, thus, contribute to pheromone divergence among populations.

*Further aspects of pheromone evolution*

Besides biotic factors, abiotic environmental conditions, like ambient temperature and humidity, can affect pheromone production (Raina 2003) and emission rate (Hock et al. 2014), and especially moisture can also influence the detectability of deposited chemical cues (Wilder et al. 2005), as well as the responsiveness of receivers (Royer & McNeil 1993). If mate choice is based on 'good genes' the condition-dependence of 'honest signals' may lead to diverging evolution among populations under differing environmental conditions (Proulx 2001, Reinhold 2011). Moreover, species isolation might be achieved by mechanisms other than pheromone signaling, like differences in the daily activity patters of sympatric males (e.g. Rundle & Nosil 2005).

Finally, one methodological point, that has to be considered when investigating the evolution of communication in general, and pheromones in particular, is that differences in pheromone composition, detected by a human observer, for instance, by GC/MS may considerably differ from what is perceived by biologically relevant receivers (Schaefer & Ruxton 2015). Thus, assessing the actual biologically relevant amount of interspecific variation in a signal requires knowledge about how the signal is perceived. In the case of pheromones, this may be achieved by comparing interspecific variation in pheromone composition not on the basis of GC/MS data, but, for example, based on coupled gas chromatography/electroantennographic detection or, ideally, behavioral assays.

In conclusion, multiple biotic and also abiotic factors can be imagined that may shape pheromone diversity in the Philanthinae. Yet, at the moment we can largely only speculate about whether these selection factors are actually relevant and how they may interact in pheromone evolution in the Philanthinae.

### 6.2.4 Physiological correlations in pheromone evolution

Given the immense interspecific variation in gland morphology among male Philanthinae (chapter 5) and the presumptive likewise large interspecific differences in pheromone composition (Weiss et al., *unpublished data*), two questions arise: (1) Does the chemical composition of a species' pheromone determine the morphology of its head glands, i.e. are there correlations between

pheromone chemistry and any aspect of gland morphology? For instance, do species that possess different types of gland cells have more complex pheromone profiles, e.g. containing more compounds with different chemical moieties, than species possessing only one type of cells? (2) Does the occurrence of different chemical pheromone compounds correlate, i.e. does the presence of certain substance classes preclude the occurrence of other classes, or does the presence of certain classes on the contrary make the co-occurrence of others more likely?

A first approach to these questions based on substance classes of tentatively identified pheromone components, however, did neither detect an obvious pattern in the co-occurrence or the exclusive presence of different chemical classes, nor in the number and complexity of different gland cell types and the occurrence of different substances (Figure 6.2). In fact, species lacking an MG (and consequently the gland cells otherwise associated with it), particularly North American *Philanthus*, even tended to have slightly more complex chemical profiles than species possessing gland cells associated with both the MG and the PPG (on average 3.0 *vs.* 2.7 different substance classes per species, excluding *n*-alkanes and alkenes that were produced by all species; Weiss et al., *unpublished data*). In stingless bees a negative correlation has been found between the phylogenetic age of a species and the diversity of substance classes in its cuticular profile (Leonhardt et al. 2013). In *Philanthus*, North American species arguably constitute the most derived phylogenetic clade (Kaltenpoth et al. 2014), possibly suggesting a similar correlation.

Two substance classes frequently detected in beewolf pheromones, namely methyl-branched HCs and ketones, might not be informative regarding a possible correlation of pheromone composition and gland morphology for two reasons: First, methyl-branched HCs and ketones are also present in the chemical profile of male *P. albopilosus*, which possess no MG and only minute PPGs with no gland cells (Figure 6.2). Second, both substance classes are also found in the PPG secretion of female Philanthini (Strohm et al. 2008, Herzner et al. 2013; chapter 3) that do likewise not possess any gland cells associated with this gland (Strohm et al. 2007, Herzner et al. 2013; chapter 2). Hence, the production of methyl-branched HCs and ketones may be independent of PPG and MG. The same likely applies to *n*-alkanes, and possibly also unsaturated HCs, which may, at least partly, be produced by oenocytes in the fat body (Soroker & Hefetz 2000, Martins & Ramalho-Ortigão 2012, Makki et al. 2014).

Some aspects of our preliminary analysis, however, might have impaired the detection of existing trend. Since primary alcohols and aldehydes are hardly distinguishable by their mass spectra without derivatization, any compound identified as either alcohol or aldehyde had consequently

been assigned to one class. Therefore, any correlation of either class with morphology and/or phylogeny would have been weakened, and a correlation between the occurrence of alcohols and aldehydes would not have been detected. Moreover, except for linear HCs, we did not distinguish between saturated and unsaturated compounds, possibly masking patterns among, for instance, saturated and unsaturated carboxylic acids or esters.

Besides the differential composition of chemical substance classes, yet another possibility to create interspecific pheromone diversity would be differences in the chain lengths of structurally related compounds. However, there is accumulating evidence that Hymenoptera may more easily discriminate between different chemical structures than different chain lengths of a molecule (Châline et al. 2005, Dani et al. 2005, van Wilgenburg et al. 2010). Possible chain length differences may, therefore, not be of prime importance regarding pheromone diversity in the Philanthinae.

In male Philanthinae, despite all interspecific variation, there are strong phylogenetic patterns in head gland morphology (chapter 5), but arguably not in pheromone chemistry (Figure 6.2). In summary, the considerable differences in pheromone composition even among closely related species and the likely absence of phylogenetic trends, support the hypothesis that pheromone chemistry in the Philanthinae may indeed mainly be driven by the need for species recognition and reproductive isolation and/or the exploitation of preexisting receiver biases in females, yet leaving open the question what may have caused the distinct differences in male gland morphology.

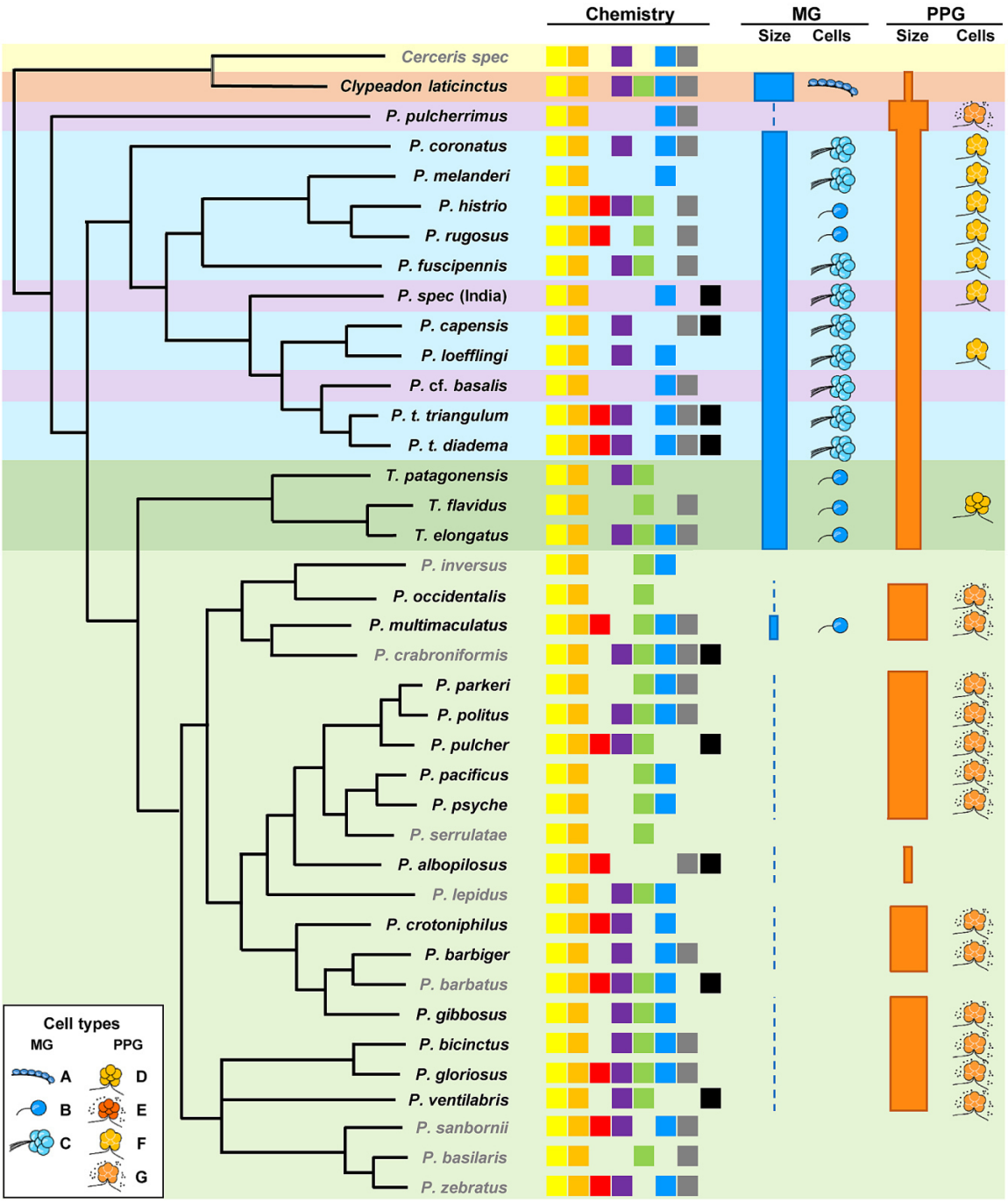


**Figure 6.2** Summary of interspecific variation in pheromone chemistry ('Chemistry'), as well as size ('Size'; bar thickness indicates relative size, dotted line indicates absence) and type of associated gland cells ('Cells') of the MG and PPG (according to Figure 5.5, chapter 5). Tentatively identified substances detected in head extracts of male Philanthinae (see text) were grouped into eight classes, based on differences in their biosynthetic pathways (Howard & Blomquist 2005, Blomquist & Bagnères 2010, Morgan 2010) following Oliveira et al. (2015) (colored boxes). Color code of chemical classes: yellow, n-alkanes; orange, alkenes; red, alkadienes; purple, carboxylic acids; green, esters; blue, alcohols and aldehydes (assigned to one class, see text); grey, ketones; black, methyl-branched alkanes. For species printed grey, only data on gland chemistry, but not morphology were available. Pictograms of cell types (labeling see key in figure): (A) single gland cells, showing end apparatuses but directly associated with the wall of the MG reservoir without canal cells; (B) single NQ-class 3 gland cells; (C) acini of several NQ-class 3 cells with bundles of conducting canals; (D) aggregations of several gland cells directly associated with very fine branches of the PPG; (E) as in (D), but interspersed with small rounded cells; (F) syncytia of secretory cells directly associated with very fine branches of the PPG; (G) syncytia as in (E), but interspersed with small rounded cells (see chapter 5 for details of male head gland morphology). Color code of phylogeographic clades (shaded background; according to Kaltenpoth et al. 2014): yellow, genus *Cerceris*; orange, genus *Clypeadon*; purple, Palearctic/Asian *Philanthus*; blue, Palearctic/Afrotropical *Philanthus*; light green, Nearctic *Philanthus*; dark green, genus *Trachypus*.

### 6.3 Concerted evolution of male and female head glands

In the Philanthinae, both males and females possess the same head glands, but while both MG and PPG are under diversifying sexual selection in males (chapter 5), at least in the Philanthini the PPG of females is under stabilizing natural selection due to their role in antimicrobial brood defense (chapter 2). Selection optima for such homologous traits often differ considerably between males and females (Lewis et al. 2011). Since the shared genome may prevent the sexes from simultaneous reaching their respective optima, there might be genetic constraints and intralocus sexual conflicts (Arnqvist & Rowe 2005, Bonduriansky & Chenoweth 2009, van Doorn 2009, Pennell & Morrow 2013), which can, for instance, mitigate sexual selection on male traits due to opposing natural selection in females (e.g. Pischedda & Chippindale 2006, Sharma et al. 2012).

In the Philanthinae, genetic constraints might account for the congruent general pattern of PPG elaboration that is observed between males and females of different philanthine lineages, i.e. small and simple glands in Cercerini and Aphilanthopsini, but larger and complex glands in Philanthini (chapters 2, 4, and 5), Yet, it remains unclear whether the initial enlargement of the PPG in the Philanthini was due to selection in males or females, i.e. whether it first became involved in pheromone production in males, subsequently facilitating its involvement in prey embalming in females or *vice versa* (see also discussion in chapter 5).

Yet, some aspects of PPG morphology of male and female Philanthinae may argue against the correlated evolution of PPG morphology. First, males of the North American *P. albopilosus* have largely reduced PPGs, which can be explained by the absence of pheromone signaling in this species (Evans & O'Neill 1988). The PPG of female *P. albopilosus*, however, is just as large and complex as in other female Philanthini (chapter 2). Second, the PPG of male *Philanthinus quattuordecimpunctatus*, a basal taxon of the tribe Philanthini, though resembling the glands of other male Philanthini in overall size and location (chapter 5), shows a structural organization that is unique among all investigated species. The PPG reservoir of male *P. quattuordecimpunctatus* possesses hardly any lumen; instead, the whole 'volume' of the gland is made up by a complex network of lamella-like branches, resulting in an enormous enlargement of the gland's surface (Figure 6.3). Yet, the PPG of female *P. quattuordecimpunctatus* again perfectly resembles the voluminous reservoirs of other female Philanthini (chapter 2). Finally, despite the overall congruent pattern in general size and complexity between tribes, in many philanthine clades, the PPG is large in both sexes, but yet much more complexly structured in males than in females,

including the association with different types of secretory cells that do never occur with female PPGs.

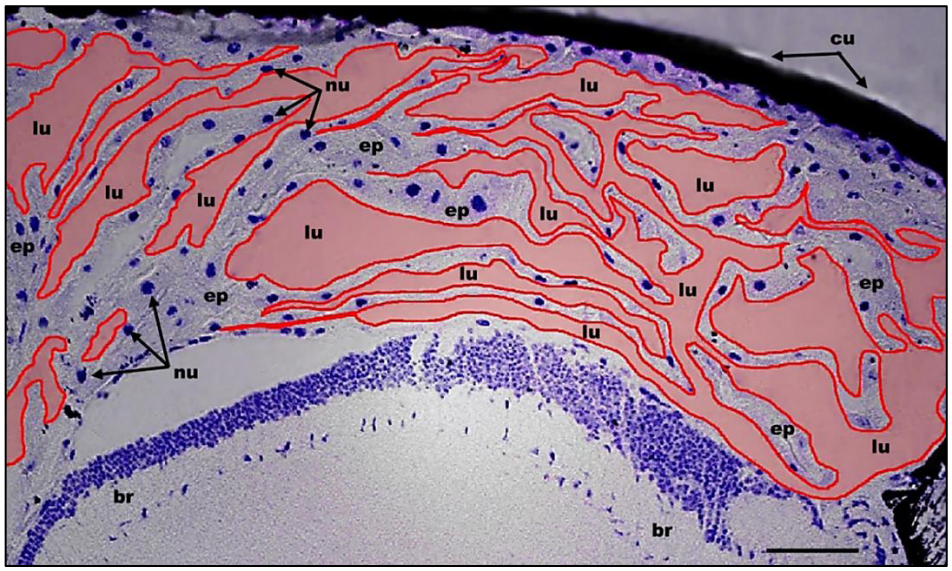


**Figure 6.3** Semithin sagittal section through the head of a male *Philanthinus quattuordecimpunctatus*. The 'volume' of the postpharyngeal gland (PPG) of *P. quattuordecimpunctatus* is made up by a complex network of thin, lamella-like branches, each bordered by a thick monolayered epithelium. Most of these loops are interconnected, forming an extremely complex network. The inner boarders of the epithelium surrounding each PPG lamella are marked in dark red; the lumen of the PPG lamellae is marked in light red. Abbreviations: br, brain; cu, cuticle of the head capsule; ep, epithelium of the PPG; lu, lumen of the PPG branches (note that not all branches of the PPG are labeled); nu, nuclei of epithelial cells. Scale bar = 50 µm.

Sexual conflicts over gland chemistry, on the other hand, seem rather unlikely. While in males it may be assumed that most pheromone components are produces by the gland cells associated with the MG (Goettler & Strohm 2008) and/or PPG, female Philanthini generally do not possess secretory cells associated with their PPGs. Rather the HCs largely constituting the females' PPG secretion are most likely produced by oenocytes in the fat body (e.g. Martins & Ramalho-Ortigão 2012, Makki et al. 2014) and sequestered into the PPG from the hemolymph (e.g. Strohm et al. 2010).

Intralocus sexual conflict can be overcome over evolutionary times. For instance, by the evolution of sex-specific gene regulation, ultimately permitting sexual dimorphic trait expression (e.g. Bonduriansky et al. 2005; see also Pennell & Morrow 2013). Therefore, genetic constraints between the sexes may nevertheless have played a role in the evolution of enlarged PPGs in the Philanthinae, despite its apparent solution in some species, for instance due to changed selection pressures in males, as in *P. albopilosus*.

## 6.4 Final conclusion

The studies presented in this PhD thesis were conceived to gain an overview over the diversity in head gland morphology and chemistry among species and sexes of the Philanthinae, thereby assessing the effect of different selection pressures. We conclusively demonstrat that in the Philanthini, but possibly not in other philanthine tribes, females employ a common antimicrobial prey protection mechanism. The use of PPG secretion in prey embalming leads to stabilizing selection on both PPG morphology and chemistry in female Philanthini. By contrast, we show that there is immense interspecific variation in the morphology of both PPG and MG among male Philanthinae and that the amount of variation in gland morphology is significantly higher in males than in females. Since male glands should be subject to sexual selection due to their role in pheromone production, we conclude that the high amount of interspecific differences in male gland morphology, and, as implied by preliminary results, likely also pheromone chemistry corroborates the often proposed diversifying nature of sexual selection.

In summary, the Philanthinae provide an ideal model for studying the evolution of shared traits under different selection regimes. By delineating the extent of interspecific variation in male and female head glands and the different evolutionary factors that may shape them, this thesis provides the basis for more detailed investigations. Further analyses of the multiple interactions that may exist between the different physiological and ecological factors shaping head gland evolution in the Philanthinae will ultimately also help to unravel the origin of the astonishing morphological diversity in male pheromone glands.

## 6.5 References


Akino T, Yamamura K, Wakamura S, Yamaoka R: Direct behavioral evidence for hydrocarbons as nestmate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Applied Entomology and Zoology* 2004, 39(3):381-387.

Albre J, Liénard MA, Sirey TM, Schmidt S, Tooman LK, Carraher C, Greenwood DR, Löfstedt C, Newcomb RD: Sex pheromone evolution is associated with differential regulation of the same desaturase gene in two genera of leafroller moths. *Plos Genetics* 2012, 8(1).

Alcock J: Male mating strategies of some philanthine wasps (Hymenoptera: Sphecidae). *Journal of the Kansas Entomological Society* 1975, 48(4):532-545.

Aldrich J, Lusby W, Marron B, Nicolaou K, Hoffmann M, Wilson L: Pheromone blends of green stink bugs and possible parasitoid selection. *Naturwissenschaften* 1989, 76(4):173-175.

Andersson MB: Sexual selection. 1st ed. Princeton University Press; 1994.

Arakaki N, Yamazawa H, Wakamura S: The egg parasitoid *Telenomus euproctidis* (Hymenoptera: Scelionidae) uses sex pheromone released by immobile female tussock moth *Orgyia postica* (Lepidoptera: Lymantriidae) as kairomone. *Applied Entomology and Zoology* 2011, 46(2):195-200.

Arnegard ME, McIntyre PB, Harmon LJ, Zelditch ML, Crampton WGR, Davis JK, Sullivan JP, Lavoue S, Hopkins CD: Sexual signal evolution outpaces ecological divergence during electric fish species radiation. *The American Naturalist* 2010, 176(3):335-356.

Arnqvist G, Rowe L: Sexual Conflict, 1st ed. Princeton University Press; 2005.

Bacquet PMB, Brattström O, Wang HL, Allen CE, Löfstedt C, Brakefield PM, Nieberding CM: Selection on male sex pheromone composition contributes to butterfly reproductive isolation. *Proceedings of the Royal Society of London B: Biological Sciences* 2015, 282(1804).

Bee MA, Micheyl C: The cocktail party problem: what is it? How can it be solved? And why should animal behaviorists study it? *Journal of comparative psychology* 2008, 122(3):235.

Beeler AE, Rauter CM, Moore AJ: Mate discrimination by females in the burying beetle *Nicrophorus orbicollis*: the influence of male size on attractiveness to females. *Ecological Entomology* 2002, 27(1):1-6.

Beye M, Hasselmann M, Fondrk MK, Page RE, Omholt SW: The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* 2003, 114(4):419-429.

Billen J: Phylogenetic aspects of exocrine gland development in the Formicidae. *Social Insects and the Environment* 1990:317-318.

Blaul B, Ruther J: How parasitoid females produce sexy sons: a causal link between oviposition preference, dietary lipids and mate choice in *Nasonia*. *Proceedings of the Royal Society of London B: Biological Sciences*. 2011, rspb20110001.

Blomquist GJ, Bagnères A-G: Insect hydrocarbons: Biology, biochemistry, and chemical ecology. 1st ed. Cambridge: Cambridge University Press; 2010.

Blows MW, Allan RA: Levels of mate recognition within and between two *Drosophila* species and their hybrids. *The American Naturalist* 1998, 152(6):826-837.

Bohart RM, Menke AS: Sphecid wasps of the world: a generic revision. 1st ed. Ithaca: University of California Press, 1976.

Bonduriansky R, Chenoweth SF: Intralocus sexual conflict. *Trends in Ecology & Evolution* 2009, 24(5):280-288.

Bonduriansky R, Rowe L, Tregenza T: Sexual selection, genetic architecture, and the condition dependence of body shape in the sexually dimorphic fly *Prochyliza xanthostoma* (Piophilidae). *Evolution* 2005, 59(1):138-151.

Boughman JW: Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* 2001, 411(6840):944-948.

Boughman JW: How sensory drive can promote speciation. *Trends in Ecology & Evolution* 2002, 17(12):571-577.

Branstetter MG, Longino JT, Ward PS, Faircloth BC: Enriching the ant tree of life: enhanced UCE bait set for genome-scale phylogenetics of ants and other Hymenoptera. *Methods in Ecology and Evolution* 2017.

Buček A, Matoušková P, Vogel H, Šebesta P, Jahn U, Weißflog J, Svatoš A, Pichová I: Evolution of moth sex pheromone composition by a single amino acid substitution in a fatty acid desaturase. *Proceedings of the National Academy of Sciences* 2015, 112(41):12586-12591.

Buchinger TJ, Wang H, Li W, Johnson NS: Evidence for a receiver bias underlying female preference for a male mating pheromone in sea lamprey. *Proceedings of the Royal Society of London B: Biological Sciences* 2013, 280(1771):20131966.

Châline N, Sandoz J-C, Martin SJ, Ratnieks FL, Jones GR: Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). *Chemical Senses* 2005, 30(4):327-335.

Chemnitz J, Jentschke PC, Ayasse M, Steiger S: Beyond species recognition: somatic state affects long-distance sex pheromone communication. *Proceedings of the Royal Society of London B: Biological Sciences* 2015, 282(1812).

Cherry EC: Some experiments on the recognition of speech, with one and with two ears. *The Journal of the acoustical society of America* 1953, 25(5):975-979.

Christy JH. Mimicry, mate choice, and the sensory trap hypothesis. *The American Naturalist* 1995, 146(2):171-181.

Clarke S, Dani F, Jones G, Morgan E, Schmidt J: (Z)-3-Hexenyl (R)-3-Hydroxybutanoate: A male specific compound in three North American decorator wasps *Eucerceris rubripes*, *E. conata* and *E. tricolor*. . *Journal of Chemical Ecology* 2001, 27(7):1437-1447.

Colazza S, Rosi MC, Clemente A: Response of egg parasitoid *Telenomus busseolae* to sex pheromone of *Sesamia nonagrioides. Journal of Chemical Ecology* 1997, 23(11):2437-2444.

Cook J: Sex determination in the Hymenoptera: a review of models and evidence. *Heredity* 1993, 71.

Cook JM, Crozier RH: Sex determination and population biology in the Hymenoptera. *Trends in Ecology & Evolution* 1995, 10(7):281-286.

Coyne J, Orr H: Speciation: Sinauer Associates Inc.; 2004.

Cremer S, Armitage SAO, Schmid-Hempel P: Social immunity. *Current Biology* 2007, 17(16):R693-R702.

Crozier RH, Dix MW: Analysis of two genetic models for the innate components of colony odor in social Hymenoptera. *Behavioral Ecology and Sociobiology* 1979, 4(3):217-224.

Dahbi A, Cerdá X, Lenoir A: Ontogeny of colonial hydrocarbon label in callow workers of the ant *Cataglyphis iberica*. *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences* 1998, 321(5):395-402.

Danci A, Schaefer PW, Schopf A, Gries G. Species-specific close-range sexual communication systems prevent cross-attraction in three species of *Glyptapanteles* parasitic wasps (Hymenoptera: Braconidae). *Biological Control* 2006, 39(2):225-231.

Danforth BN: Social insects: Are ants just wingless bees? *Current Biology* 2013, 23(22):R1011-R1012.

Dani FR, Jones GR, Corsi S, Beard R, Pradella D, Turillazzi S: Nestmate recognition cues in the honey bee: differential importance of cuticular alkanes and alkenes. *Chemical Senses* 2005, 30(6):477-489.

Debevec AH, Cardinal S, Danforth BN: Identifying the sister group to the bees: a molecular phylogeny of Aculeata with an emphasis on the superfamily Apoidea. *Zoologica Scripta* 2012, 41(5):527-535.

Domingue MJ, Starmer WT, Teale SA: Genetic control of the enantiomeric composition of ipsdienol in the pine engraver, *Ips pini*. *Journal of Chemical Ecology* 2006, 32(5):1005.

Dyer KA, White BE, Sztepanacz JL, Bewick ER, Rundle HD. Reproductive character displacement of epicuticular compounds and their contribution to mate choice in *Drosophila subquinaria* and *Drosophila recens*. *Evolution*. 2014, 68(4):1163-1175.

Elliot SL, Blanford S, Thomas MB: Host–pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London B: Biological Sciences* 2002, 269(1500):1599-1607.

Emlen DJ, Marangelo J, Ball B, Cunningham CW: Diversity in the weapons of sexual selection: horn evolution in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae). *Evolution* 2005, 59(5):1060-1084.

Endler JA. Signals, signal conditions, and the direction of evolution. *The American Naturalist* 1992, 139:S125-S153.

Evans HE, O'Neill KM. Male territorial behavior in four species of the tribe Cercerini (Sphecidae: Philanthinae). *Journal of the New York Entomological Society* 1985, 93(3):1033-1040

Evans HE, O'Neill KM: The natural history and behavior of North American beewolves. Ithaca: Cornell University Press; 1988.

Fatouros NE, Dicke M, Mumm R, Meiners T, Hilker M: Foraging behavior of egg parasitoids exploiting chemical information. *Behavioral Ecology* 2008, 19(3):677-689.

Ferveur JF: Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. *Behavior Genetics* 2005, 35(3):279-295.

Goettler W, Strohm E: Mandibular glands of male European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2008, 37(5):363-371.

Gould F, Estock M, Hillier NK, Powell B, Groot AT, Ward CM, Emerson JL, Schal C, Vickers NJ: Sexual isolation of male moths explained by a single pheromone response QTL containing four receptor genes. *Proceedings of the National Academy of Sciences* 2010, 107(19):8660-8665.

Groot AT, Horovitz JL, Hamilton J, Santangelo RG, Schal C, Gould F. Experimental evidence for interspecific directional selection on moth pheromone communication. *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103(15):5858-5863.

Hansson B, Löfstedt C, Roelofs W: Inheritance of olfactory response to sex pheromone components in *Ostrinia nubilalis*. *Naturwissenschaften* 1987, 74(10):497-499.

Haykin S, Chen Z: The cocktail party problem. *Neural Computation* 2005, 17(9):1875-1902.

Haynes KF, Yeargan KV: Exploitation of intraspecific communication systems: illicit signalers and receivers. *Annals of the Entomological Society of America* 1999, 92(6):960-970.

Hefetz A, Errard C, Cojocaru M: Heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed groups. *Naturwissenschaften* 1992, 79(9):417-420.

Hefetz A, Errard C, Chambris A, LeNegrate A: Postpharyngeal gland secretion as a modifier of aggressive behavior in the myrmicine ant *Manica rubida. Journal of Insect Behavior* 1996, 9(5):709-717.

Heimpel GE, de Boer JG: Sex determination in the Hymenoptera. *Annual Reviews of Entomology* 2008, 53:209-230.

Herzner G, Strohm E: Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Current Biology* 2007, 17:R46–7.

Herzner G, Schmitt T, Linsenmair KE, Strohm E: Prey recognition by females of the European beewolf and its potential for a sensory trap. *Animal Behaviour* 2005, 70(6):1411-1418.

Herzner G, Schmitt T, Heckel F, Schreier P, Strohm E: Brothers smell similar: variation in the sex pheromone of male European Beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implications for inbreeding avoidance. *Biological Journal of the Linnean Society* 2006, 89(3):433-442.

Herzner G, Goettler W, Kroiss J, Purea A, Webb AG, Jakob PM, Roessler W, Strohm E: Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 2007a, 36(2):123-133.

Herzner G, Schmitt T, Peschke K, Hilpert A, Strohm E: Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 2007b, 33:849–59.

Herzner G, Ruther J, Goller S, Schulz S, Goettler W, Strohm E: Structure, chemical composition and putative function of the postpharyngeal gland of the emerald cockroach wasp, *Ampulex compressa* (Hymenoptera, Ampulicidae). *Zoology* 2011a, 114(1):36-45.

Herzner G, Engl T, Strohm E: Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* 2011b, 82(2):321-328.

Herzner G, Kaltenpoth M, Poettinger T, Weiss K, Koedam D, Kroiss J, Strohm E: Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. *PLoS One* 2013, 8.

Hilker M, Bläske V, Kobs C, Dippel C: Kairomonal effects of sawfly sex pheromones on egg parasitoids. *Journal of Chemical Ecology* 2000, 26(11):2591-2601.

Hine E, Lachish S, Higgie M, Blows MW. Positive genetic correlation between female preference and offspring fitness. *Proceedings of the Royal Society of London B: Biological Sciences* 2002, 269(1506):2215-2219.

Hock V, Chouinard G, Lucas É, Cormier D, Leskey T, Wright S, Zhang A, Pichette A: Establishing abiotic and biotic factors necessary for reliable male pheromone production and attraction to pheromones by female plum curculios *Conotrachelus nenuphar* (Coleoptera: Curculionidae). *The Canadian Entomologist* 2014, 146(05):528-547.

Höglund J, Alatalo RV: Leks. Princeton Princeton University Press; 1995.

Hölldobler B, Wilson EO: The Ants. Cambridge: Harvard University Press; 1990.

Holman L, Lanfear R, d'Ettorre P: The evolution of queen pheromones in the ant genus *Lasius*. Journal of *Evolutionary Biology* 2013, 26(7):1549-1558.

Hosken DJ, Stockley P: Sexual selection and genital evolution. *Trends in Ecology & Evolution* 2004, 19(2):87-93.

Howard RW, Blomquist GJ: Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology* 2005, 50:371-393.

Iyengar VK, Eisner T: Female choice increases offspring fitness in an arctiid moth (*Utetheisa ornatrix*). *Proceedings of the National Academy of Sciences* 1999, 96(26):15013-15016.

Iyengar VK, Rossini C, Eisner T: Precopulatory assessment of male quality in an arctiid moth (*Utetheisa ornatrix*): Hydroxydanaidal is the only criterion of choice. *Behavioral Ecology and Sociobiology* 2001, 49(4):283-288.

Jackson BD, Morgan DE: Insect chemical communication: pheromones and exocrine glands of ants. *Chemoecology* 1993(4):125-144.

Johnson BR, Borowiec ML, Chiu JC, Lee EK, Atallah J, Ward PS: Phylogenomics resolves evolutionary relationships among ants, bees, and wasps. *Current Biology* 2013, 23(20):2058-2062.

Jones T, Balmford A, Quinnell R: Adaptive female choice for middle-aged mates in a lekking sandfly. *Proceedings of the Royal Society of London B: Biological Sciences* 2000, 267(1444):681-686

Juárez M, Ruiz M, Fernández P, Goane L, Villagrán M, Arce O, Armiñana A, Páez Jerez P, de la Vega M, Vera M, Groot AT: Communication interference in sympatrically occurring moth species. *Entomologia Experimentalis et Applicata* 2016, 158(1):25-33.

Kaltenpoth M, Strohm E: The scent of senescence: Age-dependent changes in the composition of the cephalic gland secretion of the male European beewolf, *Philanthus triangulum*. *Journal of Insect Science* 2006, 6.

Kaltenpoth M, Kroiss J, Strohm E: The odor of origin: kinship and geographical distance are reflected in the marking pheromone of male beewolves (*Philanthus triangulum* F., Hymenoptera, Crabronidae). *BMC Ecology* 2007, 7(11).

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E: Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(17):6359-6364.

Koedam D, Slaa E, Biesmeijer J, Nogueira-Neto P: Unsuccessful attacks dominate a drone-preying wasp's hunting performance near stingless bee nests. *Genetics and Molecular Research* 2009:690-702.

Koedam D, Morgan ED, Nunes TM, Patricio EFL, Imperatriz-Fonseca VL: Selective preying of the sphecid wasp *Trachypus boharti* on the meliponine bee *Scaptotrigona postica*: potential involvement of caste-specific cuticular hydrocarbons. *Physiological Entomology* 2011, 36(2):187-193.

Kopp A, True JR: Evolution of male sexual characters in the Oriental *Drosophila melanogaster* species group. *Evolution & Development* 2002, 4(4):278-291.

Kraaijeveld A, Ferrari J, Godfray H: Costs of resistance in insect-parasite and insect-parasitoid interactions. *Parasitology* 2002, 125(07):S71-S82.

Kroiss J, Schmitt T, Schreier P, Strohm E, Herzner G: A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 2006, 32(12):2763-2776.

Kroiss J, Bordon S, Strohm E: Hydrocarbons in the nest material of a solitary digger wasp represent a kairomone for a specialized cuckoo wasp. *Animal Behaviour* 2008, 76(5):1555-1563.

Kroiss J, Lechner K, Strohm E: Male territoriality and mating system in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae): evidence for a "hotspot" lek polygyny. *Journal of Ethology* 2010, 28(2):295-304.

Krombein KV: Biosystematic studies of Ceylonese wasps, VIII: A monograph of the Philanthidae (Hymenoptera: Sphecoidea): *Smithsonian Institution Press*, 1981.

Leary GP, Allen JE, Bunger PL, Luginbill JB, Linn CE, Macallister IE, Kavanaugh MP, Wanner KW: Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proceedings of the National Academy of Science* 2012, 109(35):14081-14086.

Leonhardt SD, Rasmussen C, Schmitt T: Genes versus environment: geography and phylogenetic relationships shape the chemical profiles of stingless bees on a global scale. *Proceedings of the Royal Society of London B: Biological Sciences* 2013, 280(1762).

Lenoir A, Fresneau D, Errard C, Hefetz A: Individuality and colonial identity in ants: the emergence of the social representation concept. In: Information processing in social insects. Springer; 1999: 219-237.

Lenoir A, Hefetz A, Simon T, Soroker V: Comparative dynamics of gestalt odour formation in two ant species *Camponotus fellah* and *Aphaenogaster senilis* (Hymenoptera: Formicidae). *Physiological Entomology* 2001, 26(3):275-283.

Lewis SM, Cratsley CK, Demary K: Mate recognition and choice in *Photinus* fireflies. *Annales Zoologici Fennici* 2004: 809-821.

Lewis Z, Wedell N, Hunt J: Evidence for strong intralocus sexual conflict in the Indian meal moth, *Plodia interpunctella*. *Evolution* 2011, 65(7):2085-2097.

Löfstedt C: Population variation and genetic control of pheromone communication systems in moths. *Entomologia Experimentalis et Applicata* 1990, 54(3):199-218.

Makki R, Cinnamon E, Gould AP: The development and functions of oenocytes. *Annual Review of Entomology* 2014, 59:405-25.

Marcillac, F, Grosjean, Y, Ferveur JF: A single mutation alters production and discrimination of *Drosophila* sex pheromones. *Proceedings of the Royal Society of London B: Biological Sciences* 2005, 272(1560):303-309.

Martin S, Drijfhout F: A Review of Ant Cuticular Hydrocarbons. *Journal of Chemical Ecology* 2009, 35(10):1151-1161.

Martins GF, Ramalho-Ortigão JM: Oenocytes in insects. *Invertebrate Survival Journal* 2012, 9:139-152.

McDaniel CA, Howard RW, Oneill KM, Schmidt JO: Chemistry of Male Mandibular Gland Secretions of *Philanthus basilaris* Cresson and *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1987, 13(2):227-235.

McDaniel CA, Schmidt JO, Howard RW: Mandibular Gland Secretions of the Male Beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera, Sphecidae). *Journal of Chemical Ecology* 1992, 18(1):27-37.

McDermott JH: The cocktail party problem. *Current Biology* 2009, 19(22):R1024-R1027.

Melo GAR: Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on the Crabronid wasps. *Scientific Papers Natural History Museum the University of Kansas* 1999(14):1-55.

Moore AJ: Genetic evidence for the "good genes" process of sexual selection. *Behavioral Ecology and Sociobiology* 1994, 35(4):235-241.

Morgan ED: Biosynthesis in insects. Cambridge: The Royal Society of Chemistry, 2004.

Niculita H, Billen J, Keller L: Comparative morphology of cephalic exocrine glands among castes of the black ant *Lasius niger*. *Arthropod Structure & Development* 2007, 36(2):135-141.

Niehuis O, Buellesbach J, Gibson JD, Pothmann D, Hanner C, Mutti NS, Judson AK, Gadau J, Ruther J, Schmitt T: Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* 2013, 494(7437):345-348.

Ohl M, Bleidorn C: The phylogenetic position of the enigmatic wasp family Heterogynaidae based on molecular data, with description of a new, nocturnal species (Hymenoptera: Apoidea). *Systematic Entomology* 2006, 31(2):321-337.

Oliveira RC, Oi CA, do Nascimento MMC, Vollet-Neto A, Alves DA, Campos MC, Nascimento F, Wenseleers T: The origin and evolution of queen and fertility signals in Corbiculate bees. *BMC Evolutionary Biology* 2015, 15(1):254.

O`Neill KM: Solitary wasps: behavior and natural history. 1st ed. Ithaca: Cornell University Press, 2001.

Ord TJ, Martins EP: Tracing the origins of signal diversity in anole lizards: phylogenetic approaches to inferring the evolution of complex behaviour. *Animal Behaviour* 2006, 71(6):1411-1429.

Ormond EL, Thomas AP, Pell JK, Freeman SN, Roy HE: Avoidance of a generalist entomopathogenic fungus by the ladybird, *Coccinella septempunctata*. *FEMS Microbiology Ecology* 2011, 77(2):229-237.

Otti O, Tragust S, Feldhaar H: Unifying external and internal immune defences. *Trends in Ecology & Evolution* 2014, 29(11):625-634.

Pennell TM, Morrow EH: Two sexes, one genome: the evolutionary dynamics of intralocus sexual conflict. *Ecology and Evolution* 2013, 3(6):1819-1834.

Peregrine D, Mudd A, Cherrett J: Anatomy and preliminary chemical analysis of the post-pharyngeal glands of the leaf-cutting ant, *Acromyrmex octospinosus* (Reich.) (Hym., Formicidae). *Insectes Sociaux* 1973, 20(4):355-363.

Pischedda A, Chippindale AK: Intralocus sexual conflict diminishes the benefits of sexual selection. *PLoS Biology* 2006, 4(11):e356.

Polidori, C., Boesi, R., Isola, F., and Andrietti, F., 2005. Provisioning patterns and choice of prey in the digger wasp *Cerceris arenaria* (Hymenoptera: Crabronidae): the role of prey size. *European Journal of Entomology* 102, 801-804.

Polidori, C., Federici, M., Papadia, C., and Andrietti, F., 2006. Nest sharing and provisioning activity of females of the digger wasp, *Cerceris rubida* (Hymenoptera, Crabronidae). *Italian Journal of Zoology* 73, 55-65.

Proulx SR. Female choice via indicator traits easily evolves in the face of recombination and migration. *Evolution* 2001, 55(12):2401-2411.

Pulawski WJ, 2017. Catalog of Sphecidae. San Francisco, SA: California Academy of Sciences. Available: http://researcharchive.calacademy.org/research/entomology/entomology_resources/hymenoptera/sphecidae/number_of_species.pdf. Accessed 5 May 2017

Raffa K, Dahlsten D: Differential responses among natural enemies and prey to bark beetle pheromones. *Oecologia* 1995, 102(1):17-23.

Raina AK: Pheromone production in corn earworm: effect of temperature and humidity. *Southwestern Entomologist* 2003, 28(2):115-120.

Rantala MJ, Jokinen I, Kortet R, Vainikka A, Suhonen J: Do pheromones reveal male immunocompetence? *Proceedings of the Royal Society of London B: Biological Sciences*. 2002, 269(1501):1681-1685.

Rantala MJ, Kortet R, Kotiaho JS, Vainikka A, Suhonen J: Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Functional Ecology*. 2003, 17(4):534-540.

Reinhold K. Variation in acoustic signalling traits exhibits footprints of sexual selection. *Evolution* 2011, 65(3):738-745.

Roelofs WL, Glover T, Tang X-H, Sreng I, Robbins P, Eckenrode C, Löfstedt C, Hansson BS, Bengtsson BO: Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proceedings of the National Academy of Science* 1987, 84(21):7585-7589.

Roelofs WL, Liu W, Hao G, Jiao H, Rooney AP, Linn CE: Evolution of moth sex pheromones via ancestral genes. *Proceedings of the National Academy of Science* 2002, 99(21):13621-13626.

Roeser-Mueller K, Strohm E, Kaltenpoth M: Larval rearing temperature influences amount and composition of the marking pheromone of the male beewolf, *Philanthus triangulum*. *Journal of Insect Science* 2010, 10.

Royer L, McNeil JN: Effect of relative humidity conditions on responsiveness of European corn borer (*Ostrinia nubilalis*) males to female sex pheromone in a wind tunnel. *Journal of Chemical Ecology* 1993, 19(1):61-69.

Rundle HD, Nosil P: Ecological speciation. *Ecology Letters* 2005, 8(3):336-352.

Ryan MJ. Sexual selection, sensory systems and sensory exploitation. *Oxford surveys in evolutionary biology* 1990, 7:157-195.

Ryan MJ. Sexual selection, receiver biases, and the evolution of sex differences. *Science* 1998, 281(5385):1999-2003.

Ryan MJ, Cummings ME. Perceptual biases and mate choice. *Annual Review of Ecology, Evolution, and Systematics* 2013, 44:437-459.

Sandland GJ, Minchella DJ: Costs of immune defense: an enigma wrapped in an environmental cloak? *Trends in Parasitology* 2003, 19(12):571-574.

Schaefer HM, Ruxton GD: Signal diversity, sexual selection, and speciation. *Annual Review of Ecology, Evolution, and Systematics* 2015, 46:573-592.

Schmid-Hempel P: Evolutionary ecology of insect immune defenses. *Annual Review of Entomology* 2005, 50:529-551.

Schmidt JO, Oneill KM, Fales HM, McDaniel CA, Howard RW: Volatiles from Mandibular Glands of Male Beewolves (Hymenoptera, Sphecidae, *Philanthus*) and Their Possible Roles. *Journal of Chemical Ecology* 1985, 11(7):895-901.

Schmitt T, Strohm E, Herzner G, Bicchi C, Krammer G, Heckel F, Schreier P: (S)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *Journal of Chemical Ecology* 2003, 29(11):2469-2479.

Schmitt T, Herzner G, Weckerle B, Schreier P, Strohm E: Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. *Apidologie* 2007, 38(2):164-170.

Seddon N, Botero CA, Tobias JA, Dunn PO, MacGregor HEA, Rubenstein DR, Uy JAC, Weir JT, Whittingham LA, Safran RJ: Sexual selection accelerates signal evolution during speciation in birds. *Proceedings of the Royal Society B-Biological Sciences* 2013, 280(1766).

Seehausen O, van Alphen JM: Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecology Letters* 1999, 2(4):262-271.

Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HD, Miyagi R, van der Sluijs I, Schneider MV, Maan ME, Tachida H: Speciation through sensory drive in cichlid fish. *Nature* 2008, 455(7213):620-626.

Sharma MD, Hunt J, Hosken DJ: Antagonistic Responses to natural and sexual Selection and the sex-specific evolution of cuticular hydrocarbons in *Drosophila simulans*. *Evolution* 2012, 66(3):665-677.

Shelly TE, Whittier TS: Lek behavior of insects. In: Choe JBC (ed.): The evolution of mating systems in insects and arachnids. Cambridge: Cambridge University Press; 1997: 273-293.

Siva-Jothy MT, Tsubaki Y, Hooper RE, Plaistow SJ: Investment in immune function under chronic and acute immune challenge in an insect. *Physiological Entomology* 2001, 26(1):1-5.

Siva-Jothy MT, Moret Y, Rolff J: Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology* 2005, 32:1-48.

Smadja C, Butlin RK: On the scent of speciation: The chemosensory system and its role in premating isolation. *Heredity* 2009, 102(1):77-97.

Soroker V, Hefetz A: Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. Journal of Insect Physiology 2000, 46:1097-1102.

Soroker V, Vienne C, Hefetz A, Nowbahari E: The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 1994, 81(11):510-513.

Soroker V, Vienne C, Hefetz A: Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera, Formicidae). *Journal of Chemical Ecology* 1995a, 21(3):365-378.

Soroker V, Hefetz A, Cojocaru M, Billen J, Franke S, Francke W: Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 1995b, 20(4):323-329.

Steiger S, Schmitt T, Schaefer HM: The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society of London B: Biological Sciences* 2011, 278(1708):970-979.

Stowe MK, Turlings T, Loughrin JH, Lewis WJ, Tumlinson JH: The chemistry of eavesdropping, alarm, and deceit. *Proceedings of the National Academy of Sciences* 1995, 92(1):23-28.

Strohm E, Lechner K: Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 2000, 59(1):183-191.

Strohm E, Laurien-Kehnen C, Bordon S: Escape from parasitism: spatial and temporal strategies of a sphecid wasp against a specialised cuckoo wasp. *Oecologia* 2001, 129(1):50-57.

Strohm E, Herzner G, Goettler W: A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 2007, 36(2):113-122.

Strohm E, Herzner G, Kaltenpoth M, Boland W, Schreier P, Geiselhardt S, Peschke K, Schmitt T: The chemistry of the postpharyngeal gland of female European beewolves. *Journal of Chemical Ecology* 2008, 34:575–83.

Strohm E, Kaltenpoth M, Herzner G: Is the postpharyngeal gland of a solitary digger wasp homologous to ants? Evidence from chemistry and physiology. *Insectes Sociaux* 2010, 57(3):285-291.

Symonds MR, Elgar MA: The mode of pheromone evolution: evidence from bark beetles. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 2004, 271(1541):839-846.

Symonds MR, Elgar MA. The evolution of pheromone diversity. *Trends in Ecology & Evolution* 2008, 23(4):220-228.

Symonds MR, Moussalli A, Elgar MA: The evolution of sex pheromones in an ecologically diverse genus of flies. *Biological Journal of the Linnean Society* 2009, 97(3):594-603.

Tabata, J, Ishikawa Y: Genetic basis to divergence of sex pheromones in two closely related moths, *Ostrinia scapulalis* and *O. zealis*. *Journal of Chemical Ecology* 2005, 31(5):1111-1124.

Thornhill R: Female preference for the pheromone of males with low fluctuating asymmetry in the Japanese scorpionfly (*Panorpa japonica*: Mecoptera). *Behavioral Ecology* 1992, 3(3):277-283.

Tragust S: External immune defence in ant societies (Hymenoptera: Formicidae): The role of antimicrobial venom and metapleural gland secretion. *Myrmecological News* 2016, 23:119-128.

van Doorn GS: Intralocus sexual conflict. *Annals of the New York Academy of Sciences* 2009, 1168(1):52-71.

van Wilgenburg E, Driessen G, Beukeboom LW: Single locus complementary sex determination in Hymenoptera: an "unintelligent" design. *Frontiers in Zoology* 2006, 3(1).

van Wilgenburg E, Sulc R, Shea KJ, Tsutsui ND: Deciphering the chemical basis of nestmate recognition. *Journal of Chemical Ecology* 2010, 36(7):751-758.

Vienne C, Soroker V, Hefetz A: Congruency of hydrocarbon patterns in Heterospecific Groups of Ants - Transfer and/or Biosynthesis. *Insectes Sociaux* 1995, 42(3):267-277.

Wagner CE, Harmon LJ, Seehausen O: Ecological opportunity and sexual selection together predict adaptive radiation. *Nature* 2012, 487(7407):366-370.

Weber MG, Mitko L, Eltz T, Ramírez SR: Macroevolution of perfume signalling in orchid bees. *Ecology Letters.* 2016, 19(11):1314-1323.

Wilder SM, DeVito J, Persons MH, Rypstra AL: The effects of moisture and heat on the efficacy of chemical cues used in predator detection by the wolf spider *Pardosa milvina* (Araneae, Lycosidae). *Journal of Arachnology* 2005, 33(3):857-861.

Zuk M, Kolluru GR: Exploitation of sexual signals by predators and parasitoids. *The Quarterly Review of Biology* 1998, 73(4):415-438.

# CHAPTER 7

## SUMMARY

The concept of evolution by natural selection, leading to the gradual adaptation of populations over time, has first been outlined by Charles Darwin. Generally, naturally selected traits evolve to maximize fitness in a given environment. Yet, already Darwin noted the existence of, often flamboyant, traits that are restricted to one sex and cannot possibly serve the fitness of their bearers under natural selection, like for example the enormous tail of a peacock. To explain the existence of such traits, Darwin invoked the concept of sexual selection, which maximizes the attractiveness (i.e. reproductive success) of their bearers, often at the expense of survival.

Generally, sexual selection is assumed to lead to rapid evolutionary changes and promote population divergence and speciation. Yet, one major drawback in the study of sexual selection is that its actual impact on the variation of a trait cannot be separated from other evolutionary forces potentially contributing to observed trait variation, like natural selection, genetic drift, or mutation.

The head glands of the digger wasp subfamily Philanthinae (Hymenoptera, Crabronidae) provide a suitable model system to study the evolution of interspecific variation in a trait under natural and sexual selection, respectively. Both the mandibular gland (MG) and the postpharyngeal gland (PPG) are present in both sexes of the Philanthinae, yet are under natural selection in females, but under sexual selection in males.

Female Philanthinae provision their offspring with paralyzed insects, mostly other Hymenoptera and Coleoptera, that are stored in subterranean brood cells, where they are exposed to a high risk of fungal infestation. Female *Philanthus triangulum* and *Trachypus elongatus* are known to embalm their prey with high amounts of unsaturated hydrocarbons (HCs) from their large and complex PPGs. This so-called 'prey embalming' reduces the condensation of water on the prey's cuticle und retards fungal growth. Based on chemical investigations of provisioned prey, the same mechanism is now proposed for *Philanthus gibbosus*, suggesting that this unique antimicrobial parental care behavior is common in all Philanthini. This hypothesis is further corroborated by the fact that all investigated females of this tribe possess uniform, complex PPGs, the secretions of

which contain predominantly unsaturated HCs. Results are mixed, however, for the other two philanthine tribes, the Cercerini and Aphilanthopsini. While all investigated species of these tribes possessing small, simple PPGs, the chemical composition of their secretions varies, with some species being dominated by methyl-branched HCs, but some possessing considerable proportions of unsaturated HCs, thus resembling the Philanthini. Taken together, at least in the Philanthini, the PPG of females is under stabilizing natural selection due to its involvement in antimicrobial prey embalming.

Males Philanthinae establish territories by scent marking with a sex pheromone, which is arguably the sole means to attract receptive females. In *P. triangulum*, the pheromone is produced and stored in the MG and PPG, and the same likely holds true for other male Philanthinae. Therefore, these glands should be under strong sexual selection. According to the theory that sexually selected traits should show high interspecific variation, there is tremendous diversity in gland morphology among male Philanthinae, regarding the occurrence of single gland reservoirs, their size and shape, as well as the type of associated secretory cells. There is, however, also an overall phylogenetic trend. Male Cercerini and Aphilanthopsini possess enormously large MGs, yet small and simple PPGs. In the Philanthini the size and complexity of the MG is reduced, while their PPG is considerably enlarged, more complex, and associated with complexes of secretory cells. In some clades of the Philanthini the MG is even lost and entirely replaced by the PPG. The amount of interspecific variation in head gland morphology is significantly higher among male than among female Philanthinae. According to preliminary results, there is likewise also marked interspecific variation in pheromone composition, with not one investigated species pair possessing the same combination of main pheromone constituents.

In summary, the Philanthinae provide an ideal model for studying the evolution of shared traits under differential selection regimes. This model allows for the direct assessment of interspecific variation caused by different selection pressures, thereby corroborating the often proposed diversifying nature of sexual selection.

# DANKE!

Zuerst möchte ich mich bei Prof. Dr. Erhard Strohm, Dr. Gudrun Herzner und allen ehemaligen und derzeitigen Mitgliedern der AG Strohm für die vielen gemeinsamen Jahre bedanken. Vielen Dank, Erhard und Guzzi, für die jahrelange tolle Betreuung und Unterstützung von der Bachelorarbeit bis zur Doktorarbeit, ich habe in dieser Zeit wirklich viel von Euch gelernt.

Danke auch an alle, ohne deren Vorarbeit und Hilfe diese Doktorarbeit überhaupt nicht möglich gewesen wäre, vor allem den vielen fleißige Bienenwolf-Sammlern, und insbesondere Margot Schilling für das unermüdliche Verarbeiten dieser Bienenwölfe in Aktenschrank-füllende Berge von Histo-Schnitten.

Außerdem danke ich Prof. Dr. Joachim Ruther für die Erstellung diverser Gutachten im Lauf der Jahre und dafür, dass ich in der AG Ruther zu jedem Anlass herzlich wollkommen war. Der gesamten AG Ruther danke für die freundliche Aufnahme, vor allem im Literaturseminar.

Für den (oft noch härteren) sportlichen Ausgleich zum harten Forscheralltag danke ich der 2. Mannschaft und allen „Jüngern des gefiederten Flugballs“ der Badmintonabteilung der SG Post/Süd Regensburg; und ganz besonders all meinen Freunden aus der Chuck-Norris-Boulder-Gruppe, meinen Schwesterfaultieren, und all den anderen Verrückten aus der Boulderwelt Regensburg. Danke für die unzähligen „Arme dick, Kopf frei“ ohne die ich die letzten Jahre nicht überlebt hätte!

Für finanzielle Unterstützung durch ein Graduiertenstipendium nach dem Bayerischen Eliteförderungsgesetz danke ich dem Universität Bayer e.V.

Der größte Dank gilt meinen Eltern Sonja und Reinhard Weiß, die mich immer mit der größten Selbstverständlichkeit unterstützt und mir so oft den Rücken freigehalten haben.